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Identification And Rescue Of Misregulated Insulin Signaling In A Drosophila Model Of Fragile X Syndrome

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Abstract
Fragile X syndrome (FXS) is an undertreated neurodevelopmental disorder characterized by low IQ and a range of symptoms including disordered sleep and autism. Although FXS is the most prevalent inherited cause of intellectual disability, its mechanistic underpinnings are not well understood. Using Drosophila as a model of FXS, we show that select expression of dfmr1 in the insulin-producing cells (IPCs) of the brain is sufficient to restore normal circadian behavior and to rescue the memory deficits in the fragile X mutant fly. Examination of the insulin signaling pathway revealed elevated levels of Drosophila insulin-like peptide 2 (Dilp2) in the IPCs and elevated insulin signaling in the dfmr1 mutant brain. Consistent with a causal role for elevated insulin signaling in dfmr1 mutant phenotypes, expression of dfmr1 specifically in the IPCs reduced insulin signaling, and genetic reduction of the insulin pathway led to amelioration of circadian and memory defects. Furthermore we showed that treatment with the FDA approved drug metformin also rescued memory. Finally, we showed that reduction of insulin signaling is required during the pupal period to improve circadian rhythmicity, but is not required until adulthood to rescue memory. Our results indicate that insulin misregulation underlies the circadian and cognitive phenotypes displayed by the Drosophila fragile X model, and thus reveal a metabolic pathway that can be targeted by new and already approved drugs to treat fragile X patients.

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ABSTRACT

IDENTIFICATION AND RESCUE OF MISREGULATED INSULIN SIGNALING IN A *DROSOPHILA* MODEL OF FRAGILE X SYNDROME

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Thomas Jongens

Fragile X syndrome (FXS) is an undertreated neurodevelopmental disorder characterized by low IQ and a range of symptoms including disordered sleep and autism. Although FXS is the most prevalent inherited cause of intellectual disability, its mechanistic underpinnings are not well understood. Using *Drosophila* as a model of FXS, we show that select expression of *dfmr1* in the insulin-producing cells (IPCs) of the brain is sufficient to restore normal circadian behavior and to rescue the memory deficits in the fragile X mutant fly. Examination of the insulin signaling pathway revealed elevated levels of *Drosophila* insulin-like peptide 2 (Dilp2) in the IPCs and elevated insulin signaling in the *dfmr1* mutant brain. Consistent with a causal role for elevated insulin signaling in *dfmr1* mutant phenotypes, expression of *dfmr1* specifically in the IPCs reduced insulin signaling, and genetic reduction of the insulin pathway led to amelioration of circadian and memory defects. Furthermore we showed that treatment with the FDA approved drug metformin also rescued memory. Finally, we showed that reduction of insulin signaling is required during the pupal period to improve circadian rhythmicity, but is not required until adulthood to rescue memory. Our results indicate that insulin misregulation underlies the circadian and cognitive phenotypes displayed by the *Drosophila* fragile X model, and thus reveal a metabolic pathway that can be targeted by new and already approved drugs to treat fragile X patients.

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*Small portions of this chapter are excerpted with modification from Monyak RE, Emerson D, Schoenfeld BP, Zheng X, Chambers DB, Rosenfelt C, Langer S, Hinchey P, Choi CH, McDonald TV, Bolduc FV, Sehgal A, McBride SM, Jongens TA. 2016. Insulin signaling misregulation underlies circadian and cognitive deficits in a *Drosophila* fragile X model. *Mol Psychiatry.* doi: 10.1038/mp2016.51 [Epub ahead of print]

Abstract:

Fragile X syndrome (FXS) is caused by loss-of-function of a single gene, *FMR1*. For most individuals affected with FXS, loss of *FMR1* occurs due to a triplet repeat expansion in the 5' untranslated region of the gene, resulting in hypermethylation of the region, and silencing of the gene. Patients with FXS show a wide range of symptoms, including intellectual disability, ADHD, aggression, autism, sleep difficulties and seizures. Affected individuals also exhibit connective tissue irregularities, a propensity toward obesity and neuroanatomical abnormalities upon autopsy. Treatments for FXS are generally supportive, and have limited efficacy.

Several animal models have been employed in the quest for better understanding of FXS. The mouse and *Drosophila* models of FXS exhibit phenotypes similar to human symptoms, including defects in memory, social behavior, sleep, and neuron morphology. The newer rat and zebrafish models of FXS show several interesting phenotypes, but have yet to substantially contribute to a better understanding of the disease.

FMR1 is an RNA binding protein that binds specifically to a large number of RNA targets. FMR1 regulates these targets by repressing them, stabilizing them and assisting with their transport to other locations in the cell. FMR1 has also been shown to play other roles in the regulation of RNA, including involvement in both the miRNA and RNA editing pathways. Furthermore, FMR1 has most recently been shown to play a critical role in the response to DNA damage.

Although it remains to be determined how loss of *FMR1* results in the physical and behavioral symptoms of FXS, defects in many signaling pathways have been implicated in FXS pathogenesis. The most studied of these pathways is the mGluR pathway, but other pathways are also involved, including the GABA pathway, the BDNF pathway, the TOR, pathway and the cAMP pathway. The involvement of these pathways in FXS leaves open the possibility that other pathways may be involved, and has stimulated our exploration of the role of the insulin signaling pathway in FXS.

Overview:

Fragile X Syndrome (FXS) is caused by loss of expression of Fragile X Mental Retardation 1 (FMR1), an RNA binding protein involved in the translation, stability and transport of up to 4% of human mRNAs (DOLEN *et al.* 2010; SANTORO *et al.* 2012). FXS is the most commonly inherited form of intellectual disability and autism, and patients also suffer from attention deficit hyperactivity disorder (ADHD), seizures, and disordered sleep (O'DONNELL AND WARREN 2002; JACQUEMONT *et al.* 2007; DOLEN *et al.* 2010). Loss of *FMR1* also results in noted neuro-anatomical defects, specifically an increased number of spines which are often elongated and immature (O'DONNELL AND WARREN 2002; JACQUEMONT *et al.* 2007).

FXS is a life-long illness that is detrimental to both the patients and their caregivers (TURK 2011; CROSS *et al.* 2016). A wide variety of drugs are used to treat the aggression, hyperactivity, anxiety and seizures associated with FXS, but no drugs are specifically approved for the treatment of the disease, and those that are most often used have questionable efficacy (SCHAEFER *et al.* 2015). Although several promising drugs have recently been tested in clinical trials, they have failed to meet FDA approval (SCHAEFER *et al.* 2015), highlighting an urgent need to obtain pharmacological targets and a more complete understanding of signaling pathways implicated in FXS pathogenesis so new potential drugs can be identified.

Molecular Basis of FXS:

As mentioned previously, loss of *FMR1* function is the causative factor in FXS. *FMR1* is a 38kb gene with 17 exons located on the X-chromosome in humans (DE VRIES *et al.* 1998). It can be alternatively spliced to encode 24 different transcripts, though it is unknown whether all are functional (DE VRIES *et al.* 1998). *FMR1* encodes a 70 kDa protein which is fairly ubiquitously expressed, but is found at particularly high levels in neurons and the testes, and high levels have also been reported in mesodermal cells and cardiac cells (DE VRIES *et al.* 1998; MCLENNAN *et al.* 2011). In normal individuals, *FMR1* has between 5 to 54 CGG repeats in its 5' untranslated region (UTR) (DE VRIES *et al.* 1998; GARBER *et al.* 2008). When this repeat region expands to over 200

repeats, it is termed a full mutation, and *FMR1* is extensively methylated and silenced (DE VRIES *et al.* 1998).

Individuals who have between 55 and 200 CGG repeats are said to have a premutation, which can expand to a full mutation within one generation (GARBER *et al.* 2008). This expansion is thought to be partially due to slippage of the replication fork and other failures during DNA synthesis, but the full mechanism of CGG repeat expansion is not entirely understood (OOSTRA AND WILLEMSEN 2009). Although the expansion from a premutation to a full mutation occurs in both the male and female germline, cells with the full mutation do not survive spermatogenesis, so only females can pass the full mutation to their offspring (GARBER *et al.* 2008). Even repeats between 45 and 54 in the 5' UTR have been documented to expend into a full mutation, though this event is unusual (GARBER *et al.* 2008).

Although the full mutation is responsible for FXS, the premutation is not entirely harmless. Males with the premutation may show symptoms similar to FXS, such as anxiety, attention problems, and even intellectual disability and autism, though these symptoms tend to be milder in presentation. Similarly, females with the premutation exhibit an increased incidence of learning disabilities and social anxiety (BOYLE AND KAUFMANN 2010). These symptoms, which are reminiscent of FXS, are thought to be due to the lower FMR1 expression present in individuals with the premutation (BOYLE AND KAUFMANN 2010). Interestingly, although FMR1 protein levels are reduced in individuals with a premutation, *FMR1* mRNA levels are greatly increased, and this phenomenon appears to lead to an entirely different disease: Fragile X-associated tremor/ataxia syndrome (FXTAS). FXTAS is a progressive neurodegenerative disease causing tremor and ataxia, and is theorized to be caused by toxicity of the repeat-containing *FMR1* transcripts (OOSTRA AND WILLEMSEN 2009). Females carrying the premutation also have a 20% chance of developing Fragile X-associated primary ovarian insufficiency, which results in premature onset of menopause (OOSTRA AND WILLEMSEN 2009). Thus in many households with a FXS patient, not only do the parents face the extremely difficult task of caring for an intellectually disabled child, in many cases the mother may have serious mental and physical health concerns of her own.

Interestingly, repeats of 200 or more CGGs do not always result in FXS. There have been reports of individuals with an unmethylated full mutation who are unaffected (D'HULST AND KOOY 2009; OOSTRA AND WILLEMSEN 2009), confirming that it is loss-of-function of *FMR1,* and not the repeat itself, that causes FXS. Further supporting this evidence, approximately 1% of those affected by FXS do not have the CGG repeat expansion. These individuals exhibit deletions or mutations in the *FMR1* gene that would be predicted to disrupt its function (GARBER *et al.* 2008). However, for 99% of those with FXS, the methylation of the full mutation is the event that causes the disease.

The methylation of the full mutation occurs early in embryogenesis after embryonic stem cells differentiate, and results in condensation of the chromatin structure around the *FMR1* gene so that its transcription ceases (D'HULST AND KOOY 2009; OOSTRA AND WILLEMSEN 2009). Clearly, preventing this critical methylation step would in effect cure FXS, but methylation-suppressing drugs are too toxic to administer to patients, and would be unlikely to affect *FMR1* methylation in enough cells to reverse FXS (OOSTRA AND WILLEMSEN 2009), even assuming that replacing *FMR1* function in childhood would be enough to reverse that developmental damage already done.

Symptoms in FXS Patients:

FXS patients exhibit a wide variety of heterogeneous symptoms, meaning that there is no one phenotype that represents FXS (BOYLE AND KAUFMANN 2010; KIDD *et al.* 2014). Symptoms vary in incidence between individuals, and factors such as gender and degree of methylation mosaicism affect severity (GARBER *et al.* 2008).

Perhaps the most striking feature of FXS is intellectual disability. Males with FXS display IQs ranging from moderately disabled (35-55) to severely impaired (20-40), while many females are less severely affected with IQs that tend to be mildly reduced to normal (DE VRIES *et al.* 1998; HAGERMAN *et al.* 2009). Because females have one normal copy of *FMR1,* they tend to exhibit less severe phenotypes than males, with severity generally correlated with the percentage of normal alleles inactivated through X-inactivation (HAGERMAN *et al.* 2009). FXS patients tend to

have difficulty with working and short-term memory, as well as impaired expressive language skills and mathematical ability (D'HULST AND KOOY 2009; BOYLE AND KAUFMANN 2010). Patients also have difficulty with executive function, leading to poor impulse control, attention problems, and difficulty with problem solving (GARBER *et al.* 2008).

Indeed, ADHD is a extremely prevalent in FXS patients, with a reported incidence as high has 93% (BOYLE AND KAUFMANN 2010). This problem generally manifests in difficulty switching between tasks, and results in individuals that may fixate on certain projects (BOYLE AND KAUFMANN 2010). Aggression is another serious problem in FXS and may develop in adulthood (DE VRIES *et al.* 1998). Although not all FXS patients show aggression, 31% of caregivers reported being injured by male patients within the past year (BAILEY *et al.* 2012), and caregivers listed the inability of FXS patients to control behavior as one of the symptoms most in need of treatment (CROSS *et al.* 2016). The incidence of aggression in FXS is an example of how genetic background influences the heterogeneity of symptoms, as aggressive behavior in FXS patients has been linked to expression of the long serotonin transporter (HAGERMAN *et al.* 2009).

Autism and Autism Spectrum Disorder (ASD) are also highly prevalent in FXS, with 60% of patients meeting the criteria for one of these diagnoses (MCLENNAN *et al.* 2011). Autistic-like behavior may be one of the first symptoms of FXS. Affected infants are sometimes extremely sensitive to touch, and may develop failure to thrive that is worsened by a high incidence of reflux and vomiting (GARBER *et al.* 2008). Even very young children with FXS exhibit poor eye contact and hand flapping, as well as continued sensitivity and aversion to touch (DE VRIES *et al.* 1998). Social anxiety is considered one of the hallmark features of FXS and often leads to avoidance and withdrawal from social situations (BOYLE AND KAUFMANN 2010).

Finally, seizures and sleep difficulties are often noted in a significant but smaller percentage of the FXS population (KIDD *et al.* 2014). Epilepsy is generally a less severe symptom of FXS as it is frequently easily controlled with pharmacological intervention and usually resolves by adulthood (KIDD *et al.* 2014). Interestingly, although only 13-18% of male FXS patients exhibit seizures, a subset of non-overtly epileptic patients display abnormal electroencephalographic

findings (HAGERMAN *et al.* 2009), suggesting the underlying neurological defects are present in a larger percentage of the population. Incidence of sleep problems is FXS is reported to be anywhere between 27% and 50% (KIDD *et al.* 2014), but the actual prevalence and full spectrum of the problem is difficult to assess, since most studies rely on reports by caregivers. A small-scale study relying on parent-recorded sleep patterns indicated that patients have more difficulty maintaining sleep and that their sleep patterns are more irregular (GOULD *et al.* 2000). A second, large-scale study based on a caregiver survey obtained similar results, with difficulty falling asleep and maintaining sleep being the most common complaints (KRONK *et al.* 2009). More serious problems such as snoring and obstructive sleep apnea have also been reported in FXS patients (KIDD *et al.* 2014). These studies generally focused on children and young adults, thus it is unknown whether these disturbances continue into adulthood. Although the reason for sleep difficulties remains a mystery, it has been reported that cortisol is more elevated in FXS patient compared to controls before bedtime (MCLENNAN *et al.* 2011). Resolving sleep problems is essential because loss of sleep has the potential to exacerbate cognitive difficulties and irritability in patients.

Given the large number of behavioral symptoms seen in FXS patients, the brains of patients have been studied, uncovering neuron morphology defects in the temporal and visual cortices (IRWIN *et al.* 2001). Examination of these regions revealed a discrepancy in the length of spines between unaffected individuals and those with FXS. Dendritic spines in FXS patients were longer, denser, and appeared immature (IRWIN *et al.* 2001).

Clearly many of the symptoms of FXS have neurological origins, but although FXS is a neurological disease, its effects range beyond the nervous system. Abnormal connective tissue accounts for a diverse array of symptoms experienced by FXS patients, ranging in severity from hyperextendable joints to mitral valve prolapsed and scoliosis (DE VRIES *et al.* 1998; KIDD *et al.* 2014). Dysfunction of connective tissues may also lead to a wide variety of gastrointestinal issues, including gastroesophogeal reflux, vomiting, constipation and diarrhea (KIDD *et al.* 2014). The Eustachian tubes of FXS patients are thought to be improperly angled so as to make

drainage difficult and leading to a high incidence of otitis media in young patients and requiring antibiotic treatment or placement of ear tubes to promote better drainage. (KIDD *et al.* 2014). Vision problems may also afflict patients, the most frequent being strabismus that makes it difficult for affected individuals to direct both their eyes toward a particular stimulus (DE VRIES *et al.* 1998). These physical complications occur in addition to the cognitive and behavioral problems exhibited by patients, thus due to poor language skills and a probable high tolerance to pain, individuals with FXS may be unable to communicate to their caregivers the nature of their suffering (KIDD *et al.* 2014), and physical discomfort due to ear or digestive problems would be expected to compound irritability and attention problems.

Finally, FXS patients present with distinctive physical changes. With increasing age, their facing develop an elongated morphology and their ears become more prominent. In males, macroorchidism is a consistent finding following puberty (BOYLE AND KAUFMANN 2010). Several studies have reported that males with FXS tend to weigh more at birth and be taller than unaffected children, but following puberty, patients are shorter than their typically-developing peers (KIDD *et al.* 2014). Patients may also have a increased propensity to obesity both as children and in adulthood, though in adulthood anti-psychotics, which are known to promote weight-gain, may be a contributing factor (MCLENNAN *et al.* 2011; KIDD *et al.* 2014). However, because of their propensity to weight gain, FXS patients are typically placed on anti-psychotics that do not affect weight as strongly, yet patients still exhibit a high incidence of obesity, suggesting that factors in the FXS metabolic background may strongly promote weight gain (MCLENNAN *et al.* 2011). Interestingly, approximately10% of FXS patients exhibit an insatiable appetite and obesity in childhood, an array of symptoms that has become known as the Prader-Willi phenotype due to its similarity to the presentation of individuals with Prader-Willi syndrome (MCLENNAN *et al.* 2011). The hyperphagia present in these patients can be so extreme their caregivers must lock the refrigerator to prevent eating of inappropriate foods (MCLENNAN *et al.* 2011). The propensity of this phenotype in FXS suggests that metabolic dysregulation is present

in FXS patients, and that even small environmental or genetic changes can tip the balance toward obesity.

Although many of the predominant symptoms of FXS are well characterized, reports of incidence differ between studies due to variations in the patient cohorts. Because females have generally milder symptoms, and have a lower occurrence rate of FXS, they are frequently underrepresented in studies and it is thus difficult to create a full picture of their symptoms. Furthermore, although FXS is a global disease, research commonly excludes most populations worldwide (TURK 2011).

Given the widespread effects of FXS, and the ubiquity of the disease, treatments for FXS are gravely needed. When a child has FXS, the entire family is affected. Caring for a child with FXS frequently financially burdens the family due to the necessity of paying for supportive care and due to loss of income from family members quitting work to care for the child (BAILEY *et al.* 2012). Although several drugs and behavioral interventions are used to treat the symptoms of FXS, nothing has been able to counteract the challenges that FXS patients and their caregivers face on a daily basis (D'HULST AND KOOY 2009). Current interventions for FXS are limited to drug treatments designed to improve the behavioral symptoms of FXS. Stimulants are often used to treat attention problems, serotonin reuptake inhibitors to treat anxiety, and anti-psychotics to stabilize mood and counteract aggression (HAGERMAN *et al.* 2009). Behavioral therapies are also used to assist FXS patients with speech and occupational therapy. However, no studies have been conducted to determine how behavioral therapies would best help those with FXS (SCHAEFER *et al.* 2015). Clearly FXS is an undertreated disease that would benefit from new therapies targeted at the molecular underpinnings of the disease.

Animal Models of FXS:

In an effort to better understand how loss of *FMR1* leads to severe behavioral and physical symptoms, several animal models have been created to better understand the functions of FMR1, and to study the neural circuits and signaling pathways involved in FXS.

Mouse Model:

A knockout (KO) mouse model was the first model of FXS to be was created. This model still exhibited a rather substantial expression of *Fmr1* mRNA, even though protein expression was entirely absent (BAKKER *et al.* 1994). Ten years later, a second *Fmr1* KO mouse line was developed that does not express *Fmr1* transcripts or protein (MIENTJES *et al.* 2006). The behavior and physical features of these mice have been examined for similarities to the symptoms displayed by FXS patients.

KO mice show multiple learning and memory defects, in keeping with the cognitive difficulties seen in those with FXS. Specifically, they exhibit defective reversal learning in the Morris water maze; although they are able to learn the location of the platform they do not adjust if the location of the platform is changed (BAKKER *et al.* 1994). Both male and female KO mice also have abnormalities in contextual and cued fear memory, and thus fail to freeze when presented with a sound or location they have been trained to associate with a shock (DING *et al.* 2014). Similarly, they show defects in passive avoidance, meaning they do not shun an area associated with a shock, although they do learn to avoid the area during training (DING *et al.* 2014). Interestingly, deficits in cued and contextual fear conditioning can be rescued by expression of *Fmr1* in only the adult neural stem cells (GUO *et al.* 2011), indicating that memory is amenable to rescue in adulthood, and that defective memory may result from failure of stem cells to properly differentiate.

Fmr1 KO mice also show evidence of social behavior defects, which parallel the high incidence of autism in FXS patients. As in patients, social deficits appear early in KO mice. Very young *Fmr1* KO pups exhibit increased ultrasonic vocalizations compared to wild-type pups, though these differences are only detectable at very discrete time points (SPENCER *et al.* 2011; LAI *et al.* 2014). Similarly, adults KO mice show differences in ultrasonic vocalizations during mating (PIETROPAOLO *et al.* 2011). Adult KO mice generally do not exhibit normal behavior in social situations. They spend less time in a mirrored chamber than wild-type mice, and initially

spend less time investigating a new mouse, though if given time, they will explore the novel mouse (SPENCER *et al.* 2005; PIETROPAOLO *et al.* 2011). This behavior bears resemblance to the often withdrawn social behavior of individuals with FXS. When paired with a female mouse, KO mice show less affiliative behavior, yet actually attempt to mount the females slightly more than wild-type mice (PIETROPAOLO *et al.* 2011), indicating that *Fmr1* KO mice tend to respond to social situations with inappropriate behavior. Supporting this observation, in some backgrounds KO mice showed increased aggression toward other mice, which has overtones of the aggression exhibited by some FXS patients (PIETROPAOLO *et al.* 2011). KO mice also generally show less dominant behavior (SPENCER *et al.* 2005). Finally, autistic-like behaviors in *Fmr1* KO mice include stereotyped behavior. For instance, KO mice show increased grooming behavior (PIETROPAOLO *et al.* 2011) and show increased marble burying (SPENCER *et al.* 2011), both phenotypes that are reminiscent of obsessive-compulsive behavior.

The mouse model of FXS does not show any detectable defects in circadian behavior (PIETROPAOLO *et al.* 2011). However, a double knockout mouse with absence of both *Fmr1* and the *Fmr1* homolog *Fxr2,* exhibits circadian rhythmicity defects, although it has a normally cycling molecular clock (ZHANG *et al.* 2008). These results suggest that *Fmr1* plays role in circadian behavior that is redundant to the role of its homolog *Fxr2*.

Perhaps the most reproducible phenotype of *Fmr1* KO mice is their susceptibility to audiogenic seizures. Following a loud noise, both male and female KO mice have seizures so serious they can result in death (YAN *et al.* 2005; DING *et al.* 2014). This phenotype parallels the propensity of FXS patients to exhibit epilepsy. KO mice also have other phenotypes paralleling human symptoms. Both male and female KO mice are frequently reported to be hyperactive (BAKKER *et al.* 1994; PIETROPAOLO *et al.* 2011; DING *et al.* 2014), which mirrors the extremely high incidence of ADHD seen in humans with FXS. KO mice also exhibit macroorchidism (BAKKER *et al.* 1994), a trait also present in humans.

Interestingly, there are some phenotypes displayed by KO mouse that seem to run counter to those seen in FXS patients. First, there is a noted decrease in anxiety-like behaviors in

KO mice. Specifically, they are more likely to enter an open field than wild-type mice (YAN *et al.* 2005) and they display more exploratory behavior (BAKKER *et al.* 1994). KO mice also show increased prepulse inhibition (PPI) whereas patients show decreased PPI (PIETROPAOLO *et al.* 2011; DING *et al.* 2014). These phenotypes that seem to be the opposite of the behaviors displayed by FXS patients remind us that no model will perfectly recapitulate a human disease, but that even opposing behaviors suggest that defects may occur in a similar pathway, and thus that study of these behaviors still has the potential to elucidate underlying disease pathogenesis.

Like human patients, the KO mouse also shows defects in neural morphology. Several studies found that dendritic spines in the layer V pyramidal cells in the cerebral cortex of KO mice are longer, thinner, and twisted, as well as potentially being present in increased number (COMERY *et al.* 1997; IRWIN *et al.* 2002). Interestingly, though these defects were present at 1 week, they virtually disappeared after 4 weeks (NIMCHINSKY *et al.* 2001), suggesting that that even brief changes during development could foretell behavioral defects later in life. KO mice also exhibit defects in neurogenesis, as well as neuron morphology. Examination of KO mice revealed a cell autonomous necessity of FMR1 for proper differentiation of neurons, with KO mice showing increased differentiation toward astrocytes and decreased differentiation toward neurons. Strikingly, this differentiation defect occurs in adult neural stem cells (GUO *et al.* 2011), indicating that loss of FMR1 is not strictly a developmental problem, but rewires the brain throughout life. Interestingly, *FMR1* KO mice also exhibit defects in synaptic plasticity, specifically showing enhanced long-term depression in the hippocampus (HUBER *et al.* 2002).

A major caveat of studying the *Fmr1* KO mouse is that their phenotypes are highly susceptible to genetic background. Several studies have analyzed how KO phenotypes vary between backgrounds, and often find that a phenotype will be strong in one background but completely absent from another (PIETROPAOLO *et al.* 2011; SPENCER *et al.* 2011). This strong effect of background on KO phenotypes should not be altogether surprising, since FXS patients have varied genetic backgrounds and their symptoms are extremely heterogeneous (KIDD *et al.* 2014). To further complicate the situation, sometimes phenotypes are not reproducible, even in

the same background (PIETROPAOLO *et al.* 2011; SPENCER *et al.* 2011). Indeed, it has been established that the identity of the experimenter can have an influence on behavior in mice (SORGE *et al.* 2014). These factors can at times make it difficult to reproduce behavioral phenotypes. Since no animal disease model is perfect, it is therefore wise to explore disease in multiple models.

Drosophila Model:

The fruit fly model of FXS is based on loss-of-function of the *dfmr1* gene. *Dfmr1* is the sole *Drosophila* homolog of *FMR1* and the *FMR1* homologs *FXR1* and *FXR2* (WAN et al. 2000). dFMR1 contains two KH domains and an RGG box like FMR1, and is able to bind RNA (WAN *et al.* 2000). Furthermore, both proteins have a similar expression pattern, with dFMR1 localized mainly to the cytoplasm of cells, and exhibiting a particularly high expression in the nervous system, but no expression in glia (WAN *et al.* 2000; ZHANG *et al.* 2001; MORALES *et al.* 2002). Amazingly, this simple fly is able to recapitulate many of the phenotypes seen in human FXS patients, and has proven to be a valuable tool in understanding the function of FMR1 and in elucidating the underlying pathology behind FXS.

One of the first behavioral phenotypes seen in *dfmr1* mutant flies was arrhythmic circadian behavior. Wild-type flies show a rhythmic activity pattern in complete darkness even without outside cues, but *dfmr1* mutant flies fail to maintain rhythmicity (DOCKENDORFF *et al.* 2002; INOUE *et al.* 2002; MORALES *et al.* 2002). Interestingly, these defects do not occur due to abnormal cycling of the molecular components of the central clock, as *dfmr1* mutant flies show normal cycling of *tim* and *per* mRNA and protein, suggesting that the defect lies in the clock output pathway (DOCKENDORFF *et al.* 2002; INOUE *et al.* 2002). A similar phenotype, eclosion rhythm, which normally leads to most eclosion occurring predominantly before lights on (KONOPKA AND BENZER 1971), has been reported to be normal in mutants in some cases (DOCKENDORFF *et al.* 2002) and abnormal in others (INOUE *et al.* 2002; MORALES *et al.* 2002). Interestingly, the *dfmr1* circadian phenotype is very similar to the *Fmr1* and *Fxr2* double knockout mouse phenotype

suggesting that since *Drosophila* do not have the *Fxr1* and *Fxr2* genes (WAN *et al.* 2000), *dfmr1* plays the role of *Fxr1* and *Fxr2* as well as that of *Fmr1*. This peculiarity of the fly may give them an advantage over the mouse model, since the lack of redundancy may accentuate some roles of *Fmr1* that might be missed in a murine model of FXS. Although humans with FXS do have the benefit of the redundant function of *Fxr1* and *Fxr2*, it is possible that the loss of *FMR1* may cause FXS patients to be more sensitive to perturbation of their circadian rhythms, and slower to acclimate to perturbation.

Sleep is also disrupted in *dfmr1* mutants. Mutant flies sleep for longer periods of time and are more resistant to arousal (BUSHEY *et al.* 2009; VAN ALPHEN *et al.* 2013). It should be noted that this phenotype is the opposite of the typically described symptoms of sleep disorder in FXS, in which patients are described as having difficulty maintaining sleep (GOULD *et al.* 2000; KRONK *et al.* 2009). This discrepancy could be due to the different parameters used to measure fly sleep and human sleep. Interestingly, the *dfmr1* mutants were reported to show more intense sleep during the day (VAN ALPHEN *et al.* 2013) and FXS patients were reported to be tired during the day (KRONK *et al.* 2009), perhaps suggesting that the time of preferred sleep is shifted.

Dfmr1 mutants also exhibit defects in learning and memory, which parallels the cognitive difficulties faced by FXS patients. *Dfmr1* mutants demonstrate memory deficiencies in two different memory assays. In the olfactory conditioning assay, flies are trained to associate one of two odors with a shock stimulus. When the flies are subsequently given a choice between the two odors, those that remember will move toward the odor unassociated with the shock (TULLY *et al.* 1994). Long-term memory, as well as immediate recall memory (henceforth referred to as learning) is defective in *dfmr1* mutants trained using this assay. Specifically, protein synthesis-dependent long-term memory is affected. (BOLDUC *et al.* 2008).

In a second assay, the conditioned-courtship paradigm, a male fly is trained with an unreceptive female fly. During the training, his courtship attempts decrease as he experiences rejection, a process referred to as learning (SIEGEL AND HALL 1979). It is important to note that this decrease is not due to fatigue, as a male paired with a receptive female will not decrease his

courtship advances (GAILEY *et al.* 1984). If a male is then paired with a receptive female, his courtship toward this female should be decreased compared to an untrained male (SIEGEL AND HALL 1979). *Dfmr1* mutant flies show defects in immediate recall memory, short-term memory and long-term memory in this assay, but do show learning (MCBRIDE *et al.* 2005; CHOI *et al.* 2010). Interestingly, a subsequent study has shown that with age, *dfmr1* mutants no longer show learning, although their wild-type counterparts continue to display it (CHOI *et al.* 2010). These results may reflect some evidence in human patients that cognitive performance declines with age (TURK 2011). The ability of *dfmr1* mutants to learn but not remember in the conditioned courtship assay parallels the ability of KO mice to learn but not remember in the passive avoidance assay (DING *et al.* 2014), suggesting that there may be conserved cognitive features between the mouse and *Drosophila* models of FXS.

Dfmr1 mutants also display defects in social behavior. Mutant males exhibit abnormally low courtship levels when paired with a female fly (DOCKENDORFF *et al.* 2002). These flies initiate courtship as often as wild-type males, however shortly after the initiation, they cease courting (DOCKENDORFF *et al.* 2002). Interestingly, male *Fmr1* KO mice also have defects in mating behavior with females mice, manifesting as abnormal vocalizations and inappropriate interaction behavior (PIETROPAOLO *et al.* 2011). Whether the courtship song of *dfmr1* mutants is abnormal has never been addressed, though it could be an interesting source of inquiry.

Dfmr1 mutants have subsequently been identified as having aberrant social behavior in a second assays that measures the amount of time *dfmr1* mutants spend near other flies (BOLDUC *et al.* 2010). It was determined that *dfmr1* mutants spend less time interacting with other flies than wild-type flies (BOLDUC *et al.* 2010), indicating that *dfmr1* mutants exhibit a deficit in social behaviors unrelated to courtship. Finally, *dfmr1* mutants have been noted to show excessive grooming behavior that becomes more evident with age, a phenotype that is reminiscent of obsessive-compulsive behavior (TAUBER *et al.* 2011). Interestingly, excessive grooming behavior was also seen in KO mice (PIETROPAOLO *et al.* 2011), giving further evidence of conservation of the *dfmr1* gene.

Other behavioral phenotypes have also been noted in *dfmr1* mutants. One group noted very low activity levels in mutants, which could have been due to serious lethality problems in which only 1% of flies eclosed from the pharate adult stage (MORALES *et al.* 2002). Other groups report no problems with lethality of *dfmr1* mutants (DOCKENDORFF *et al.* 2002). Mutant larvae also show abnormal crawling behavior. These larvae were observed to execute more turns than wild-type larvae and would not crawl in a straight line for as long (XU *et al.* 2004). Adult flies also have locomotor abnormalities, were noted to display severe defects in flying (ZHANG *et al.* 2001).

Similar to humans and *Fmr1* KO mice, *Dfmr1* mutants have neurological abnormalities. *Dfmr1* mutants exhibit overelaboration and increased synaptic boutons in the neuromuscular junction (NMJ) (ZHANG *et al.* 2001). Mutants display increased branching of the LNv neurons, which are part of the circadian clock (DOCKENDORFF *et al.* 2002) and display abnormal branching of the DC neurons (MORALES *et al.* 2002). *Dfmr1* mutants also demonstrate aberrant structure of the mushroom bodies (MICHEL *et al.* 2004), a prominent neural structure in the brain that is essential for learning and memory. In *dfmr1* mutants, the normally separate beta lobes of the mushroom body fuse together. Interestingly, this crossover phenotype is highly dependent on genetic backgrounds (MICHEL *et al.* 2004), thus demonstrating how easily FXS phenotypes can be influenced by background mutations.

Other Models:

Although the mouse and fly are the primary models used to understand how *FMR1* affects behavior and brain development, in recent years several new models have emerged that may be able to complement those that currently exist. The zebrafish *Fmr1* KO was made in 2009, and was found to have defective morphology of the Rohon-Beard neurites, but no other noted physical phenotypes (DEN BROEDER *et al.* 2009). Further exploration of the zebrafish KO discovered that the fish also had reduced anxiety in novel environments and increased exploration (NG *et al.* 2013; KIM *et al.* 2014), similar to the phenotype of the KO mouse (BAKKER *et al.* 1994; YAN *et al.* 2005). One study noted hyperactivity (NG *et al.* 2013), while the other did

not (KIM *et al.* 2014). Finally, brain slices from KO fish were found to have reduced LTP and increased LTD (NG *et al.* 2013), once again closely echoing the KO mouse (HUBER *et al.* 2002).

A second new FXS model organism is the KO rat. This model was created using zinc-finger nucleases, and it was confirmed that these animals do not produce FMR1 (HAMILTON *et al.* 2014). These mutant rats show reduced play behavior, but in contrast to the *Fmr1* KO mouse, did not exhibit deficits in memory, anxiety, reversal learning or fear conditioning (HAMILTON *et al.* 2014; TILL *et al.* 2015). However, the KO rats did display problems with associative recognition memory, which is hippocampus dependent (ENGINEER *et al.* 2014; TILL *et al.* 2015). One interesting study showed that KO rats have decreased brain activity in response to speech compared to wild-type rats. However, they were able to distinguish consonants and vowels as well, if not better than, wild-type rats, so the "speech" defect appears to be in not the hearing or learning of sounds, but in the processing (ENGINEER *et al.* 2014). The rat KO also has physiological problems. They show increased protein synthesis and enhanced LTD (TILL *et al.* 2015), similar to KO mice. They also show dendritic morphology defects mirroring those of the mouse KO (TILL *et al.* 2015). The advent of the rat KO will allow the exploration of novel phenotypes, not seen in mouse or fly models, and could act as another method to analyze the defects underlying FXS.

Functions of FMR1:

Given the mirroring of FXS symptoms in four different animal models, it is clear that FMR1 has a critical role in maintenance of the neural morphology and behavior. This observation brings us to the question of how FMR1 functions on a molecular level.

FMR1 is expressed at high levels in neurons, but is not present in glia when expression is examined in the rat brain (FENG *et al.* 1997b). It is also specifically located in synaptoneurosomes (FENG *et al.* 1997b), suggesting it has an important role in signaling between neurons. FMR1 is mostly located in the cytoplasm of cells, though small amounts of FMR1 are found in the nucleus, and are able to shuttle in and out of this central organelle (EBERHART *et al.* 1996; WILLEMSEN *et al.*

1996; CORBIN *et al.* 1997; FENG *et al.* 1997a). Indeed, FMR1 has a nuclear localization sequence as well as a nuclear export sequence (EBERHART *et al.* 1996), thus it is capable of moving freely between the cytoplasm and nucleus.

RNA Binding:

In the cytoplasm, FMR1 co-sediments with polyribosomes in an RNA-dependent manner (EBERHART *et al.* 1996; KHANDJIAN *et al.* 1996; WILLEMSEN *et al.* 1996; CORBIN *et al.* 1997; FENG *et al.* 1997b). Indeed, FMR1 has the functional domains of an RNA-binding protein. FMR1 has two RNP K homology (KH) domains as well as an RGG box, all domains known to be important for binding RNA in other proteins (SIOMI *et al.* 1994). KH domains bind to kissing complex RNAs, and dFMR1 has been shown to bind to these RNAs as have FXR1 and FXR2 (DARNELL *et al.* 2009). The KH domains are known to be critical role to FMR1 function because a single point mutation, I304N, in one of these domains can cause severe FXS (DE BOULLE *et al.* 1993). However, some groups have reported that this mutation does not completely abolish the ability of FMR1 to bind to RNA (BROWN *et al.* 1998). Nevertheless, other groups report that the I304N mutation results in decreased RNA binding (SIOMI *et al.* 1994) and mutations in the KH domain abolish the association of FMR1 with polyribosomes (DARNELL *et al.* 2005), suggesting that its presence may disrupt RNA binding enough to jeopardize FMR1 function. Tellingly, making the equivalent mutation in either KH domain of the *dfmr1* gene resulted in decreased courtship behavior and reduced circadian rhythmicity (Banerjee et al. 2007), phenotypes that mimic *dfmr1* loss-of-function. These observations imply that the KH domain is critical for FMR1's ability to bind RNA, and that this ability is essential for its role in promoting normal behavior.

The RGG box, the other functional domain in FMR1 is also important for RNA binding. The RGG box is known to bind to G-quartet structure in RNA, and it alone is sufficient to bind some RNAs (DARNELL *et al.* 2001). Furthermore, loss of the RGG box results in weakened RNA binding (BROWN *et al.* 1998). Both FMR1 and dFMR1 are methylated at the RGG box, resulting in decreased binding to G-quartet RNAs (STETLER *et al.* 2006), suggesting that methylation of FMR1

may be a mechanism of regulating its activity. However, absence of the RGG does not prevent FMR1 from associating with polyribosomes, suggesting that the RGG box may be important for other RNA binding functions of FMR1 that do not involve the translational machinery (DARNELL *et al.* 2005). Interestingly, a recent study was unable to identify the G-quartet structure as a preferred FMR1 binding site, indicating that the RGG box is either not a major binding motif, or that it is bound in combination with other signal sequences (ANDERSON *et al.* 2016).

The ability of FMR1 to bind RNA is critical to its function, and interestingly, it does not always bind RNA alone. FMR1 interacts with a long list of other RNA binding proteins to perform a variety of functions. Specifically, FMR1 has been shown to associate with LARK, a circadian protein (SOFOLA *et al.* 2008), with NUFIP, a protein important for RNA maturation (BARDONI *et al.* 2003), with insulin-like Growth Factor II mRNA binding protein I (IMP1), CYFIP, a protein important for cytoskeletal maintenance (SCHENCK *et al.* 2003), and Caprin, another polyribosome-associated protein (EL FATIMY *et al.* 2012). Its interactions with these proteins and others allow it to specifically regulate its RNA targets.

Naturally, the identities of the RNAs bound by FMR1 are of great interest. It is estimated that FMR1 binds as many as 4% of all fetal RNAs in humans (ASHLEY *et al.* 1993) and that these RNAs are specifically targeted by FMR1 (ASHLEY *et al.* 1993; BROWN *et al.* 1998). The first study to search for the mRNA targets of FMR1 used a microarray to interrogate mRNAs associated with FMR1 that was immunoprecipitated from mouse brains. Over 400 targets were identified (BROWN *et al.* 2001). A second early experiment used the cDNA-SELEX system to identify target sequences in humans (CHEN *et al.* 2003). However, these targets did not show much overlap with the previous experiment, raising the question of how reliable either list was.

A third experiment used HITS-CLIP to discover RNA targets of FMR1 (DARNELL *et al.* 2011). This experiment identified a large number of targets, including many that are associated with autism spectrum disorder. This list included *NLGN3, NRXN1, SHANK3, PTEN, TSC2*,*NF1*, as well as many RNAs previously identified by BROWN *et al.* (2001), and many novel targets (DARNELL *et al.* 2011). A fourth experiment used PAR-CLIP to identify RNA Recognition Elements

bound by FMR1. Interestingly, this experiment also identified many genes known to be affected in autism as FMR1 targets (ASCANO *et al.* 2012). Together, these experiments begin to uncover why FXS results in the symptoms it does, but it also raises the question of how to use a long list of genes to understand how *FMR1* loss of function causes FXS. First we need to understand how FMR1 regulates its targets.

Repression of Translation:

The next important question is how FMR1 regulates its target RNAs. As is hinted by its association with ribosomes, it has the ability to regulate translation, often by repressing it. In the KO mouse, some RNAs show a higher level of translation (ZALFA *et al.* 2003), suggesting that their repressor is missing. FMR1 *in vitro* has been observed to repress translation of *polyA* mRNA without affecting RNA stability (LI *et al.* 2001) indicating that FMR1 does not promote decay of its targets, but rather prevents them from being translated. In *dfmr1* mutants, several dFMR1 targets show increased translation without any change in mRNA level. The first of these is *futsch*¸ the *Drosophila map1b* homolog (ZHANG *et al.* 2001). In *dfmr1* mutants Futsch is more highly expressed and leads to defects in the neuromuscular junction (NMJ) (ZHANG *et al.* 2001). The translation of the Futsch homolog, MAP1B, is also negatively regulated by FMR1 in mammals (LU *et al.* 2004). In *Drosophila,* dFMR1 also acts as a repressor of translation for *chickadee*, the *Drosophila profilin* homolog, and an important regulator of the actin cytoskeleton (REEVE et al. 2005). Arc, yet another mRNA involved in cytoskeletal maintenance is also repressed by FMR1 (NIERE *et al.* 2012), indicating how FMR1 may regulate neural structure through regulation of its targets. *FMR1* may partly function to regulate local translation. In the *Drosophila* ovaries, dFMR1 suppresses *orb* translation, but also interacts with the Orb protein to regulate local translation of mRNA (COSTA *et al.* 2005). Thus one of the major ways FMR1 represses translation is by binding to its specific targets and silencing them.

FMR1 may also repress translation by repressing the translation of targets that promote translation. Both *mtor* and *tsc2* were identified as targets that FMR1 negatively regulates (ASCANO

et al. 2012). Since mTOR stimulates translation, loss of *FMR1* would result in not only an increase of translation of its specific target mRNAs, but also a more global increase in translation due to the lack of repression of mTOR. FMR1 can also repress non-specific translation by its interaction with CYFIP, a 4E-BP-like protein that binds to the translation initiation factor eIF4E to sequester it from the translational machinery (Napoli et al. 2008). At the synapses, stimulation with DHPG causes CYFIP to disassociate with eIF4E, allowing translation to commence (NAPOLI *et al.* 2008).

FMR1 is also able to very specifically repress the translation of its target RNAs. When FMR1 associates with polyribosomes, it stalls translation of the bound target RNA (DARNELL *et al.* 2011). For FMR1 to function properly, it must be able disassociate from the polyribosomes so translation can resume. The key to this ability is phosphorylation. After FMR1 is synthesized, it is phosphorylated at Serine 499, resulting in phosphorylation of surrounding serines (CEMAN *et al.* 2003). As long as FMR1 is phosphorylated, it is able to associate with polyribosomes and stall translation. External signaling can trigger dephosphorylation of FMR1, resulting in dissociation with the ribosomes, and allowing translation to proceed (CEMAN *et al.* 2003; LEE *et al.* 2011).

Other Regulation of RNA by FMR1

Importantly, FMR1 does not always repress translation when it binds to RNA. It has also been shown to be involved in stabilization, transport, and in some cases promotion of translation. In *Drosophila*, dFMR1 has been shown to regulate stability of *pickpocket1 (ppk1)* mRNA (XU *et al.* 2004). In humans, FMR1 activates *nitric oxide synthase 1 (nos1)* translation by binding to a G-quartet motif (KWAN *et al.* 2012). Interesting, this activation occurs during early brain development and synaptogenesis and reduced *nos1* in humans is associated with ADHD and aggression (KWAN *et al.* 2012). However, mice do not have a G-quartet motif in their *nos1* mRNA, and FMR1 is not known to bind *nos1* in mice (KWAN *et al.* 2012). Likely testing more of the many transcripts of FMR1 will reveal a longer list of RNA targets that are not regulated by the “classic” repression of translation.

FMR1 also plays a role in the transportation and localization of mRNAs. It may be important for suppressing the translation of mRNAs until they arrive at the location where they will be translated. In *Drosophila,* association of dFMR1 with *Drosophila* lethal giant larvae, results in formation of granules that contain several specific mRNAs (ZARNESCU *et al.* 2005). It is likely that this association is important for cell polarity and division (ZARNESCU *et al.* 2005). FMR1 also associates with Staufen-containing stress granules along with various motor proteins (VILLACE *et al.* 2004). Likely this association is important for localization of mRNAs to different part of the cell or neuron (VILLACE *et al.* 2004). Indeed, in S2 cells, dFMR1 and RNA granules move throughout the cell in a kinesin and dynein-dependent manner (LING *et al.* 2004). dFMR1 associates with both dynein heavy chain and kinesin heavy chain (LING *et al.* 2004). Even more intriguing, dFMR1 is necessary for efficient transport of these granules. In the absence of dFMR1, the granules oscillate back and forth more frequently and fail to move smoothly in one direction (ESTES *et al.* 2008). In mice, FMR1 has been shown to be important for transport of its target mRNAs into the dendrites of neurons following DHPG stimulation (DICTENBERG *et al.* 2008).

Involvement in miRNA Pathway:

FMR1 also has a well-established yet still mysterious role in the miRNA pathway. FMR1 is known to associate with Ago1, Ago2, Dmp68 and Dicer in the RISC complex in both mice and in *Drosophila* (CAUDY *et al.* 2002; ISHIZUKA *et al.* 2002; JIN *et al.* 2004). However, the full role of FMR1 is this complex is not entirely understood. Ago1 has been shown to be essential for dFMR1 to promote apoptosis in the fly eye (JIN *et al.* 2004), suggesting that perhaps the miRNA complex may sometimes be important for the way FMR1 binds to RNA and regulates its targets. However, in spite of having an established presence in RISC, FMR1 does not seem to necessary for miRNA mediated degradation to occur (CAUDY *et al.* 2002).

Of interest, however, is a study showing that FMR1 sometimes associates with miRNAs. These RNAs are miR-125b and miR-132, which have a role in dendritic spine morphology which is dependent on FMR1 (EDBAUER *et al.* 2010). Interestingly, one of the miRNAs, miR-125b,

targets *NR2A*, which is also a FMR1 target (EDBAUER *et al.* 2010), suggesting that FMR1 may sometimes regulate its target mRNAs with the help of miRNAs and RISC. In another instance, FMR1 was shown to bind to the 3'UTR of *psd-95* mRNA in KO mice and to repress its translation in conjunction with miR-125a and AGO2 (MUDDASHETTY *et al.* 2011). FMR1 binding was dependent on its phosphorylation (MUDDASHETTY *et al.* 2011). These findings that FMR1 and RISC may function together to regulate some RNA targets.

Interaction with ADAR and RNA editing:

FMR1 is now known to associate with ADAR, the adenosine deaminase responsible for adenosine to inosine RNA editing in both *Drosophila* larvae and zebrafish embryos (BHOGAL *et al.* 2011; SHAMAY-RAMOT *et al.* 2015; DEFFIT AND HUNDLEY 2016). In *Drosophila*, ADAR acts downstream of FMR1 to control the architecture of the neuromuscular junction, and ADAR must maintain its RNA editing activity to perform this role (BHOGAL *et al.* 2011).

Interestingly, absence of FMR1 appears to have implications for ADAR's RNA editing activity in both *Drosophila* and zebrafish. In *Drosophila,* transcripts from *dfmr1* mutants showed alterations in RNA editing levels, although these alterations resulted in both increases and decreases in editing, suggesting that FMR1 may act to balance RNA editing efficiency (BHOGAL *et al.* 2011). In zebrafish, ADAR levels were increased in the absence of *Fmr1*, and RNA editing was slightly increased (SHAMAY-RAMOT *et al.* 2015). These results show that FMR1 is important for multiple aspect of RNA regulation, and suggest the possibility that FXS could partially result from abnormal RNA editing.

Function in response to DNA damage:

FMR1 has recently been shown to play an unexpected role in the DNA damage response pathway. Intriguingly, replication stress and DNA damage induced by chemicals or irradiation recruits FMR1 to the nucleus (ZHANG *et al.* 2014). Furthermore, FMR1 associates with chromatin

during times of possible DNA stress, such as during meiosis and when single-stranded breaks occur (ALPATOV *et al.* 2014).

The evidence suggests that FMR1 has a critical role in the response to DNA damage. *Fmr1* mutant cells showed decreased γH2A.X foci formation when single-stranded DNA breaks were induced (ALPATOV *et al.* 2014). Similarly, replication stress-induced recruitment of γH2Av, the *Drosophila* equivalent of γH2A.X, was reduced in *Drosophila* cells not expressing dFMR1 (ZHANG *et al.* 2014).

Altogether, FMR1 is an incredibly versatile protein that functions in both the nucleus and the cytoplasm to respond to DNA damage, and to regulate RNA translation, processing and stability.

Signaling Pathways Affected by loss of FMR1:

mGluR Signaling:

Although many pathways are likely regulated by FMR1, the first to be identified was the mGluR pathway. The mGluR theory of FXS was introduced in 2004, and suggested that misregulated mGluR signaling could be responsible for all FXS phenotypes. Specifically, this theory suggested that loss of FMR1 could cause mGluR dysfunction by lifting the break on signaling normally maintained by the presence of FMR1 (BEAR *et al.* 2004). Indeed, mGluR dysfunction does seem to be a major contributor to the FXS phenotype. Supporting the idea that mGluR signaling is increased in FXS, it was found that *dfmr1* mutants are susceptible to high concentrations of glutamate in food and cannot survive in such conditions (CHANG *et al.* 2008).

FMR1 plays an important role in support and regulation of mGluR signaling. Following activation of the mGluR pathway, FMR1 suppresses LTD, and if FMR1 is overexpressed LTD can actually be abolished entirely. (HOU *et al.* 2006). Supporting the role of FMR1 in regulating LTD, following mGluR stimulation FMR1 is found near the synapses in rat brains (WEILER *et al.* 1997). As has been detailed earlier, FMR1 represses translation by stalling polyribosomes (DARNELL *et al.* 2011). Upon mGluR stimulation, PP2A dephosphorylates FMR1, resulting in restart of

translation (NIERE *et al.* 2012). After FMR1 is dephosphorylated, it is ubiqutinated and degraded in the proteosome through a PP2A-dependent mechanism. (NALAVADI *et al.* 2012). This entire process is essential for translation of FMR1's mRNA targets (NALAVADI *et al.* 2012).

Another mechanism of mGluR misregulation may occur through altered binding to Homer proteins, a group of proteins that function as scaffolding proteins. Normally mGluR5 associates with both the long and short forms of Homer, but in *Fmr1* KO mice, this balance is disrupted and mGluR5 associates predominantly with the short isoform even though the long isoform continues to be present at normal levels (RONESI *et al.* 2012). Interestingly, deleting the short *Homer* gene rescued defects in translation and audiogenic seizures, and decreased anxiety-like behavior of *Fmr1* mutants (RONESI *et al.* 2012).

The central tenant of the mGluR Theory of FXS is that reduction of mGluR signaling should rescue FXS phenotypes (BEAR *et al.* 2004). Indeed, many phenotypes of *FMR1* mutants can be rescued by administration of a pharmacological mGluR antagonist, or even through genetic mutation of the mGluR gene. In *Drosophila,* mGluR antagonists were shown to rescue courtship-based memory defects in STM as well as LTM defects in olfactory conditioning, mushroom body cross-over and naïve courtship defects (MCBRIDE *et al.* 2005; BOLDUC *et al.* 2008). mGluR antagonists also rescued age related learning deficits (CHOI *et al.* 2010). Interestingly, the antagonists were able to rescue memory after acute administration in adulthood (MCBRIDE *et al.* 2005), suggesting that cognitive deficits of FXS may still be amenable to rescue with the right pharmacological agent. mGluR antagonists have also been shown to rescue NMJ structural deficits in *dfmr1* mutants (PAN *et al.* 2008) and a dmGlurA null mutation rescued hyperexcitability seen in *dfmr1* mutants (REPICKY AND BROADIE 2009).

mGluR inhibitors have also been explored in mice, with many promising results. First, it has been shown that mGluR inhibitors rescue audiogenic seizures as well as the increased exploratory behavior seen in KO mice (YAN *et al.* 2005). mGluR inhibitors also rescued the reduced number of mRNA granules seen in KO brains (ASCHRAFI *et al.* 2005). Finally, it has been shown that even treatment with mGluR inhibitors after the onset of the phenotype can rescue

inhibitory avoidance defects, dendritic spine density, and partially rescue macroorchidism (MICHALON *et al.* 2012).

Clearly mGluR antagonists have potential as a therapy for FXS, and indeed they have reached clinical trials. However, those trials were unsuccessful and have been stopped (SCHAEFER *et al.* 2015). Explanations for this lack of success have ranged from clinical measures simply being not sensitive enough to detect changes in children or adults given the drugs, to the possibility that individuals become tolerant of the drugs over time, an effect that was noted previously in mice (YAN *et al.* 2005).

Other Signaling Pathways:

The mGluR Theory of FXS (BEAR *et al.* 2004) discounts the idea that other signaling pathway are involved in FXS. Although loss of FMR1 clearly affects glutamate signaling, FMR1 has many diverse mechanisms of regulating mRNA. It seems extremely unlikely that only one signaling pathway underlies all defects seen by loss of *Fmr1*.

GABA is one signaling pathway affected by loss of *Fmr1.* Its possible role in FXS is unsurprising, since GABA is the inhibitor that puts the break on the glutamate excitation, thus the two pathways function together. GABA agonist drugs were found to rescue glutamate toxicity in *dfmr1* mutant *Drosophila*, as well as to rescue relevant phenotypes such as mushroom body crossover and depressed male courtship (CHANG *et al.* 2008). Furthermore, it has been shown that both *dfmr1* mutants and mouse *Fmr1* KOs have lowered expression of several GABA-A subunits (D'HULST *et al.* 2006; CURIA *et al.* 2009). Also, in *Fmr1* KO mice, GABA-A currents are decreased (CURIA *et al.* 2009) and in the striatum, KO mice have increased GABA release, but reduced GABA synapses (CENTONZE *et al.* 2008), suggesting that the increased release of GABA might be compensation for decreased GABA synapse number. GABA-A and GABA-B agonists have also been shown to rescue audiogenic seizures in KO mice (PACEY *et al.* 2009; HEULENS *et al.* 2012).

Bone-derived neurotrophic factor (BDNF) signaling is also disrupted in the mouse *Fmr1* KO. BDNF has been shown to be increased in the hippocampus and decreased in the cortex of mice lacking FMR1. Also, the BDNF receptor, TrkB, exhibits increased expression in neural progenitor cells (LOUHIVUORI *et al.* 2011). However, the increased BDNF expression in the hippocampus disappears after 4 months (UUTELA *et al.* 2012). Even more interesting, a mouse heterozygous for *Bdnf* and null for *Fmr1* exhibits both improved and exacerbated behavior compared to *Fmr1* KO mice and *Bdnf* heterozygote mice. The double mutant had decreased performance in the water maze and contextual fear memory, but rescue of hyperactivity. Also, KO of *Fmr1* rescue the obesity of the *Bdnf* heterozygote. However, not all of these effects were not very strong or convincing (UUTELA *et al.* 2012). Still, these results imply that BDNF signaling in *Fmr1* mutants is not disrupted in the same direction in all parts of the brain.

Another pathway that has been shown to be dysregulated is the TOR pathway. TOR forms a complex that stimulates protein synthesis and controls many other pathways. Interestingly, there are several pieces of evidence that suggest that TOR activity is increased in *Fmr1* KO mice. Brains of KO mice show increased levels of phosphorylated and activated TOR, and decreased inhibition of cap-dependent translation (SHARMA *et al.* 2010). In *Fmr1* KO animals, it has been shown that reducing S6K1, a downstream factor in the TOR pathway, rescues excessive protein synthesis, excessive LTD, increased weight gain in pups, and macroorchidism. Reducing S6K1 in KO mice also rescued the dendritic spine morphology, social interaction deficits and reversal learning deficits (BHATTACHARYA *et al.* 2012).

Another pathway disrupted in FXS is cAMP signaling. cAMP disruption was reported in 2002, when it was shown that the normal cAMP oscillations are attenuated in *dfmr1* mutants (DOCKENDORFF *et al.* 2002). *Fmr1* KO mice have been shown to have reduced cAMP levels in brains and platelets, and cAMP is also reduced in FXS neurons and *dfmr1* mutant heads (KELLEY *et al.* 2007). In *Drosophila* and mice, PDE-4 inhibitors have been used to see if increasing cAMP levels can cause improvements in behavior and other pathologies. Indeed, it was shown that PDE-4 inhibitors were able to rescue short term memory and immediate recall deficits in the

conditioned courtship assay, as well as immediate recall memory in the olfactory conditioning assay. Also, LTD was rescued in hippocampal slices after PDE-4 inhibitor administration (CHOI *et al.* 2015).

Even more disrupted pathways have emerged. mAChR antagonists were able to rescue audiogenic seizures and marble burying in *Fmr1* KO mice, though there was some possibility that the drug had off-target effects (VEERARAGAVAN *et al.* 2011). Inhibition of GSK-3β rescued fear memory and rebalances neural differentiation in *Fmr1* KO mice (GUO *et al.* 2012). Finally, it has been shown that minocycline, a matrix metalloprotease inhibitor, rescues decreased ultrasonic vocalizations made by KO mice when in a mating situation (ROTSCHAFER *et al.* 2012).

Altogether, the implication of dysfunction of multiple pathways in FXS suggests that to understand the disease, we need to understand how these pathways interact to cause behavioral deficits. We also need to consider how best to treat FXS as a disease of many pathologies.

Hypothesis:

Clearly FXS is a disease of many heterogeneous symptoms, a disease of many mRNA targets, and a disease of many signaling pathway defects. We believe that although many of these pathways have been uncovered, there may be others yet to be revealed. We hypothesize that insulin signaling is abnormal in *dfmr1* mutant flies, and that this misregulated signaling pathway underlies behavioral phenotypes in the fly model of FXS.

Here we show that *dfmr1* expression in a small group of neurons, the insulin-producing cells (IPCs), is essential for normal circadian behavior and memory. We also show that insulin signaling is disrupted in the brains of *dfmr1* mutants and that by reducing insulin signaling either genetically or pharmacologically, we can rescue behavioral dysfunction. Finally, we show that properly regulated insulin signaling during the pupal period is critical for rhythmic behavior, but that normalized insulin signaling is not required until adulthood to rescue memory. These findings suggest a new pathway involved in FXS, and reveal new possible therapeutic targets.

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Chapter 2: Insulin signaling in *dfmr1* mutants is misregulated

*Portions of this chapter are excerpted with modification from Monyak RE, Emerson D, Schoenfeld BP, Zheng X, Chambers DB, Rosenfelt C, Langer S, Hinchey P, Choi CH, McDonald TV, Bolduc FV, Sehgal A, McBride SM, Jongens TA. 2016. Insulin signaling misregulation underlies circadian and cognitive deficits in a *Drosophila* fragile X model. *Mol Psychiatry.* doi: 10.1038/mp2016.51 [Epub ahead of print]

Abstract:

Previous studies of *dfmr1* mutant flies suggested that the circadian behavior defect exhibited by these flies defective clock output circuitry. To identify neurons that might be involved in the circadian output circuit, we conducted a screen for groups of neurons in which *dfmr1* expression was sufficient to rescue circadian behavior in an otherwise *dfmr1* null background. We found that expression of *dfmr1* in the clock neurons was insufficient to restore circadian rhythmicity, however expression of *dfmr1* in the insulin-producing cells (IPCs) completely ameliorated the circadian defect. Intriguingly, expression of *dfmr1* in this small group of neurons was also able to restore courtship-based and olfactory-based memory in *dfmr1* mutants.

Since the IPCs are the primary site of insulin production in *Drosophila,* we examined levels of *Drosophila* insulin-like peptide 2 (Dilp2) in the brains of *dfmr1* mutants. Interestingly, we found that Dilp2 levels were increased in the IPC cell bodies of *dfmr1* mutants, and furthermore, that downstream markers of insulin signaling such as PI3K activity and pAkt were also increased in the brains of *dfmr1* mutants, suggesting that insulin signaling is increased upon loss-of-function of *dfmr1.* We also determined that restoration of *dfmr1* to only the IPCs was sufficient to ameliorate the insulin signaling defect, indicating that the defect is cell non-autonomous in nature. These results suggest that altered insulin signaling could play a role in the behavioral phenotypes of *dfmr1* mutants.

Introduction:

One of the distinctive characteristics of FXS is low IQ and resulting problems with memory (GARBER *et al.* 2008). However, the symptoms of FXS are wide-ranging and heterogeneous and include, among other things, a high incidence of sleep disorders (KIDD *et al.* 2014).

As discussed in Chapter 1, *dfmr1* mutants have defects in behavior that mimic the symptoms of FXS patients. Specifically, *dfmr1* mutants have defects in circadian rhythmicity (DOCKENDORFF *et al.* 2002; INOUE *et al.* 2002; MORALES *et al.* 2002) and two forms of memory (MCBRIDE *et al.* 2005; BOLDUC *et al.* 2008). *Dfmr1* mutants have learning and long-term memory (LTM) defects in the olfactory conditioning paradigm (BOLDUC *et al.* 2008), in which flies are conditioned to associate a shock with an odor, so they will subsequently avoid the conditioned odor during testing (TULLY *et al.* 1994). *Dfmr1* mutants also have defects in immediate recall memory and short-term memory (STM) in the conditioned courtship assay (MCBRIDE *et al.* 2005), in which they are trained to associate courtship of a female with rejection, resulting in depressed courtship levels during testing with a receptive female fly (SIEGEL AND HALL 1979). Since sleep and cognitive symptoms are prevalent in FXS (KIDD *et al.* 2014), we can use the fly model to better understand the molecular and neurological pathways that underlie these conditions.

Intense research in the FXS field has been focused on the mGluR signaling pathway as the possible cause of FXS (BEAR *et al.* 2004). Indeed, our lab and others have shown that mGluR inhibition ameliorates the memory deficits of *dfmr1* mutant flies (MCBRIDE *et al.* 2005; BOLDUC *et al.* 2008), just as mGluR inhibition improves memory and other phenotypes in *Fmr1* knockout mice (MICHALON *et al.* 2012). However, while mGluR inhibition improved both social behavior and mushroom body morphology, it did not improve circadian behavior in *dfmr1* mutant flies (MCBRIDE *et al.* 2005). These results indicate that circadian arrthmicity might be independent of mGluR signaling.

We therefore sought to determine what defects might explain the abnormal circadian behavior of *dfmr1* mutant flies. Under normal circumstances, circadian behavior is controlled by a

central clock in the fly brain composed of around 150 neurons (NITABACH AND TAGHERT 2008). In these neurons, *per* and *tim* RNAs are transcribed in a cyclic manner, induced by a heterodimer of the circadian proteins Clock and Cycle. Once they are translated, the Per and Tim proteins heterdimerize and repress the Clock/Cycle heterodimer (LIM AND ALLADA 2013). This cycle spans 24 hours and can be set by light so that the clock can synchronize with daylight (PESCHEL AND HELFRICH-FORSTER 2011). Importantly, disruption of this clock is known to lead to arrhythmicity in flies (PESCHEL AND HELFRICH-FORSTER 2011). Although a dysfunctional molecular clock might explain arrythmic circadian behavior in *dfmr1* mutants, previous studies revealed that the circadian defect is due to faults in output pathways of the clock, and not in the central clock itself (DOCKENDORFF *et al.* 2002; MORALES *et al.* 2002).

Here we identify a new group of cells involved in the circadian output circuit in which *dfmr1* expression is important for normal circadian behavior and memory. These cells, the insulin-producing cells (IPCs), are the chief insulin synthesizing cells in *Drosophila*, and are located in the *pars intercerebralis* (PI) region of the brain (NASSEL *et al.* 2013). The identification of these cells as having a role in circadian arrhythmicity in *dfmr1* mutants led us to query whether they were involved in other phenotypes. Interestingly, we determined that *dfmr1* expression in the IPCs was sufficient to restore STM in the conditioned courtship paradigm, as well as learning and LTM in the olfactory conditioned paradigm.

Examination of the defects in these cells due to loss of *dfmr1* expression led us to investigate the insulin signaling pathway in *dfmr1* mutant flies. We found that Drosophila insulin-like peptide 2 (Dilp2) levels were increased in the cell bodies of the IPCs, so we examined insulin signaling in the Kenyon cell region of the brain to determine if the brain experienced increased insulin signaling. We found that phosphoinositide 3-kinase (PI3K) activity, a downstream readout of insulin signaling, was increased in this region of the brain, as was Akt activity, indicating that insulin signaling was high in the brains of *dfmr1* mutant flies.

Finally, to determine if loss of *dfmr1* in the IPCs might be the origin of this increased insulin signaling in the brain, we restored *dfmr1* to the IPCs and examined Akt activity in the

brain. The discovery that expression of *dfmr1* in the IPCs restored normal Akt activity led us to conclude that *dfmr1* expression in the IPCs has a cell non-autonomous effect on insulin signaling in the rest of the brain. Altogether, we discovered a group of neurons essential to normal behavior in *dfmr1* mutants, and present a new signaling pathway that is disrupted by loss of *dfmr1* expression.

Results:

Mapping studies indicate role for IPCs in circadian output

To identify the mechanism through which *dfmr1* regulates circadian behavior, we utilized the binary Gal4/UAS system (BRAND AND PERRIMON 1993) to determine the spatial requirement of *dfmr1* expression for normal circadian rhythms. We first verified that *dfmr1* activity is required in the nervous system by restoring pan-neuronal *dfmr1* expression in an otherwise null mutant fly. We found that *dfmr1* mutants containing both the pan-neuronal driver, *elav-Gal4*, and *UAS-dfmr1* transgenes displayed an increased number of rhythmic flies, as well as significantly improved relative Fast Fourier Transform (FFT) values, which measure the strength of circadian rhythmicity of one genotype relative to rhythmic strength of wild-type controls (Figure 2-1). These results indicate that expression of *dfmr1* in the nervous system is sufficient to rescue the circadian defect observed in *dfmr1* null flies.

Circadian studies have defined a fly pacemaker circuit that consists of approximately 150 neurons (NITABACH AND TAGHERT 2008). To determine if *dfmr1* activity within this circuit is sufficient to restore normal circadian behavior, we introduced several *Gal4* drivers that direct expression to subsets of clock cells into the *dfmr1* mutant background. First we used *pdf-Gal4* and *cry-Gal4* to express *dfmr1* in the ventral lateral neurons (LN_v), which have been shown to display morphological defects in the *dfmr1* mutants and are essential for maintenance of free-running rest:activity patterns (HELFRICH-FORSTER AND HOMBERG 1993; HELFRICH-FORSTER 1998; RENN *et al.* 1999; DOCKENDORFF *et al.* 2002; REEVE *et al.* 2005). However, expression of *dfmr1* in the LN_vs of *dfmr1* mutants did not improve rhythmicity in the mutant flies (Figure 2-2a). Similarly,

using *per-Gal4* or *tim-Gal4* drivers to more broadly direct *dfmr1* throughout the circadian clock circuit failed to discernibly rescue circadian behavior (Figure 2-2b).

We then explored the possibility that *dfmr1* activity was required outside the established circadian clock circuit by testing *Gal4* drivers that direct expression to various neural groups. Interestingly, we found that expression of *dfmr1* using two independent *dilp2-Gal4* drivers led to a significant rescue of rhythmicity that was comparable to the rescue obtained with pan-neuronal *dfmr1* expression (Figure 2-3a & b). The *dilp2-Gal4* drivers direct expression to 14 insulin-producing neurons located in the PI region of the brain (Figure 2-4a), a neuroendocrine center known to be important for circadian regulation in other insects and proposed to be important in *Drosophila* (HELFRICH-FORSTER *et al.* 1998; KANEKO AND HALL 2000). These 14 neurons, termed the IPCs, are the sole insulin-producing neurons in the fly brain (RULIFSON *et al.* 2002) (Figure 2-4b). Our results indicate that providing *dfmr1* function to the IPCs is sufficient to rescue the circadian defect displayed by *dfmr1* mutants.

Expression of dfmr1 in the IPCs is important for several forms of memory

We then interrogated whether the IPCs were involved in other behavioral phenotypes of *dfmr1* mutants. We first queried whether IPC-directed expression of *dfmr1* could rescue STM in the conditioned courtship paradigm. Examination of *dfmr1* mutant flies in which *dfmr1* was expressed in the IPCs revealed that STM was restored (Figure 2-5a & b). These results led us to query how *dfmr1* expression in the IPCs affected olfactory-based memory. Restoration of *dfmr1* to the IPCs of otherwise *dfmr1* null flies resulted in significant improvement in both learning and protein synthesis-dependent LTM (Figure 2-6a & b). These findings established that the IPCs were involved in a circuit essential for both circadian behavior and memory. Furthermore, it suggested that *dfmr1* expression in the IPCs was important for proper functionality of these 14 neurons.

Dilp2 Protein is elevated in dfmr1 mutant IPCs

Given that expression of *dfmr1* in the IPCs rescues the circadian and memory defects, we explored the possibility that insulin signaling was misregulated in *dfmr1* mutants. Examination of the levels of the major insulin-like peptide Dilp2 consistently revealed significantly elevated Dilp2 protein in the cell bodies and axons of *dfmr1* mutant versus control IPCs (Figure 2-7a & b, Figure 2-8). Conversely, *dilp2* mRNA levels are not increased in *dfmr1* mutant flies compared to controls (Figure 2-9a), suggesting that Dilp2 protein levels are increased by a post-transcriptional mechanism. We also examined mRNA levels of the other two *dilp* genes produced in the IPCs, *dilp3* and *dilp5,* but did not see any convincing increases in either compared to controls (Figure 2-9b & c). Interestingly, *dilp5* mRNA levels were significantly increased compared to the w^- wild-type control, but were unchanged compared to the *WTrescue* control (Figure 2-9c), leaving us unable to determine if the increase is credible or due to background effects.

It should be noted that an increase in Dilp2 levels in the cell bodies of the IPCs does not necessarily signify an overall increase in insulin signaling. Several groups observed increased Dilp2 in the cell bodies of the IPCs concurrent with an overall decrease in insulin signaling, seeming to indicate that release of Dilp2 was being repressed (KAPLAN *et al.* 2008; GEMINARD *et al.* 2009). It was therefore essential for us to determine how the downstream insulin signaling pathway was affected in *dfmr1* mutants.

PI3K Activity is increased in dfmr1 mutants

In the *Drosophila* insulin signaling pathway, Dilps activate the insulin receptor (InR), resulting in InR auto-phosphorylation and stimulating subsequent phosphorylation of PI3K. Once activated, PI3K converts the membrane phospholipid phosphatidylinositol (4,5)-biphosphate (PIP2) into phosphatidylinositol (2,4,5)-triphosphate (PIP3) (TELEMAN 2010). We examined the components of this pathway to better understand how insulin signaling could be affected in *dfmr1* mutant flies.

To determine how the altered expression levels of Dilp2 in the IPCs impacts insulin signaling in the *dfmr1* mutant brain, we first examined PI3K activity using a ubiquitously expressed GFP-pleckstrin homology (PH) domain reporter. This reporter protein localizes to the plasma membrane when membrane PIP3 levels increase upon activation of PI3K (BRITTON *et al.* 2002). Although the GFP-PH reporter is seen broadly throughout the brain, we visualized and quantified reporter protein localization in the cells on the posterior surface of the brain in the mushroom body calyx region because this area of the brain contains a large number of easily imaged cell bodies. When we examined *dfmr1* mutant brains expressing GFP-PH, we found that the reporter protein was more strongly localized to the plasma membrane in *dfmr1* mutant versus control brain neurons (Figure 2-10a & b), indicating that PI3K activity levels are elevated in the examined region of the *dfmr1* mutant brain. Subsequent assessment of GFP-PH reporter protein expression by Western analysis revealed that reporter expression was similar in both *dfmr1* mutant and control heads (Figure 2-11a & b). These results confirmed that the changes observed by immunofluorescence reflect alterations in PI3K activity rather than changes in reporter protein expression levels.

Akt phosphorylation is increased in dfmr1 mutants

We next examined Akt phosphorylation at S505, the site of its activation in *Drosophila*, using whole mount immunostaining and confocal analysis. Akt is recruited to the membrane by increased PIP3 levels caused by increased PI3K activity, and is then phosphorylated and activated (TELEMAN 2010). Inspection of p-S505-Akt levels in *dfmr1* mutants revealed increased levels of p-S505-Akt in *dfmr1* mutant brains compared to controls (Figure 2-12a & b). Altogether, these results indicate that insulin signaling is elevated in the brains of *dfmr1* mutants.

Intriguingly, we observed that p-S505-Akt signal was mainly confined to the mushroom body calyx region of the brain in both *dfmr1* mutants and controls. To determine if the Kenyon cells, the mushroom body cell bodies, were stained by p-S505-Akt, we expressed a *UAS-CD8-GFP* transgene driven by the *30y-Gal4* driver, which drives expression in the Kenyon cells, and

stained the brains with anti-p-S505-Akt. Imaging of these brains revealed that the entire Kenyon cell calyx showed p-S505-Akt signal along with a small group of neighboring cells (Figure 2-13). This finding suggests that insulin signaling in the Kenyon cells is particularly high compared to other regions of the brain.

To test whether abnormal function of the IPCs could be responsible for increased insulin signaling in the *dfmr1* brain, we examined p-S505-Akt staining in *dfmr1* mutants in which *dfmr1* was expressed in the IPCs. Incredibly, the increased concentration of p-S505-Akt seen in *dfmr1* mutants was significantly decreased by directed expression of *dfmr1* to the IPCs of *dfmr1* mutant brains (Figure 2-14a & b), indicating that the expression of *dfmr1* in these cells corrects the elevated insulin signaling in *dfmr1* mutant brains in a cell non-autonomous manner. It is also notable that p-S505-Akt levels are noticeably increased in the *dfmr1* mutants containing the *UAS-dfmr1* transgene alone compared to the *dfmr1* mutants containing the *dilp2-Gal4* transgene alone (Figure 2-14a & b). This discrepancy could be due to the effects of a leaky UAS construct that produces some *dfmr1*, suggesting that dFMR1 may have dose-dependent effects on p-S505-Akt levels. Western analysis of total Akt expression in heads revealed that overall Akt expression remained constant, indicating that the increase in p-S505-Akt levels in *dfmr1* mutants is due to increased Akt activity rather than changes in Akt expression levels (Figure 2-15).

Discussion:

In this chapter, we show that insulin signaling is misregulated in *dfmr1* mutants, at least partially due to lack of dFMR1 expression in the major insulin-producing cells of fly, the IPCs. Specifically, we show that expression of *dfmr1* in the IPCs is sufficient to rescue circadian behavior to the same degree as pan-neuronal expression of *dfmr1*. In contrast, expression of *dfmr1* in any combination of the clock neurons was unable to achieve significant rescues of circadian behavior. Together with the finding that expression of *dfmr1* in the IPCs rescues both olfactory and courtship-based memory, these findings indicate that expression of *dfmr1* in the IPCs is essential for their function in a normal fly.

Complementing the finding that the IPCs are an important component of the circuits that are disrupted in the *dfmr1* mutant fly, we found that insulin signaling is increased in the brains of *dfmr1* mutants. Specifically, we found that Dilp2 levels are elevated in the cell bodies of the IPCs in *dfmr1* mutants, and that cells in the brain show increased PI3K activity and p-S505-Akt, both markers of increased insulin signaling. Finally, we showed that expression of *dfmr1* in the IPCs rescued this increase in brain-wide insulin signaling in a cell non-autonomous manner.

Our finding that the expression of *dfmr1* in the IPCs is important for normal behavior is interesting and critical. First, it identifies a new group of cells that are involved in the circadian and memory circuitry of the *Drosophila* brain, and it will be important to gain a better a idea of how this circuitry works, and what other neurons are involved. This question could be partially addressed by further examining the many already established neurons that provide input onto the IPCs (NASSEL *et al.* 2013), to see which of these inputs may be important for circadian behavior, and which may be important for memory. When neural groups post-synaptic to the IPCs are identified, it will be interesting to determine how these neurons are involved in behavior.

Our results also determined that *dfmr1* expression in the IPCs was sufficient to rescue both circadian behavior and memory in *dfmr1* mutants. These results raise the obvious question of whether *dfmr1* expression in the IPCs is necessary for these two behaviors. To address the question of necessity, we need to exclude *dfmr1* from the IPCs and determine if the absence of *dfmr1* from only these cells abolishes either memory or circadian behavior. Unfortunately, this conceptually simple experiment has proven difficult to realize in reality. We have attempted knockdown of *dfmr1* in the IPCs using various *UAS-dfmr1-RNAi* lines, but have been unable to achieve adequate lowering of *dfmr1* levels to replicate behavioral deficits. Indeed, this failure is unsurprising given that even a small amount of dFMR1 is sufficient to maintain behavioral stability. A second approach to this question would be to use the Gal80 suppressor of Gal4 (MCGUIRE *et al.* 2004) under the control of the *dilp2* promoter and paired with the *elav-Gal4* and *UAS-dfmr1* transgenes in a *dfmr1* mutant background to express *dfmr1* in all neurons except the IPCs. If it was determined that *dfmr1* was necessary in the IPCs to promote circadian rhythmicity

and memory, we would know that the IPCs truly are a crucial group of cells for these behaviors. If we found that *dfmr1* expression in the IPCs was not necessary for normal circadian behavior and memory, we would want to look for the other groups of cells in which *dfmr1* expression might also be important. Of course, the third possibility in this scenario is that *dfmr1* expression in the IPCs might be necessary for one behavior but not the other. This finding could elucidate the nature of the circuits governing both behaviors, and would give us a clue as to how the circuitry governing circadian behavior and memory overlaps and diverges. Importantly, discovering a new circuit involved in circadian behavior or memory would contribute to our knowledge of the neurological basis for these behaviors.

In this chapter, we assessed Dilp2 levels in the IPCs. Dilp2 is one of three Dilps synthesized in these cells (IKEYA *et al.* 2002), so ideally we would examine the levels of all three Dilps. Our failure to conduct this assessment was due to our inability to obtain antibodies for Dilp3 and Dilp5. Once good antibodies are available, it will be important to examine levels of all Dilps released by the IPCs to obtain a more complete picture of insulin signaling in *dfmr1* mutants. Although we were unable to examine protein levels of Dilp3 and Dilp5, we were able to assess mRNA levels. While we did not detect convincing increases in either of these transcripts in *dfmr1* mutants, *dilp5* expression was significantly increased compared to one wild-type control, but not compared to the other. While this result could indicate an actual increase in *dilp5* mRNA levels in *dfmr1* mutants, it more likely is the result of background divergence between the w^- and *WTrescue* stocks.

After identifying the IPCs as being a central and important place of *dfmr1* function in the brain, we attempted to identify the mechanism by which loss of dFMR1 in the IPCs resulted in behavioral defects. Since a central role of the IPCs is the synthesis and release of insulins, we examined levels of Dilp2, the most abundant of the Dilps, in the cell bodies of the IPCs and discovered increased Dilp2 in the cell bodies. However, as discussed previously in this chapter, increased Dilp2 in the cell bodies does not necessarily foretell increased release of Dilp2 (KAPLAN *et al.* 2008; GEMINARD *et al.* 2009). To address this issue, we examined downstream components

of the insulin pathway in the brain, but a more direct way to address the issue would be to examine insulin release into the hemolymph. Although we tried multiple techniques to assess Dilp2 levels in the hemolymph, we were hampered by our inability to obtain a Dilp2 antibody that was specific to Dilp2. Fortunately, a new method of assessing Dilp2 levels has been developed that involves expression of a HA and FLAG-tagged Dilp2 in a *dilp2* null background (PARK *et al.* 2014). This genetic tool should bypass the difficulty of finding a Dilp2-specific antibody and will thus allow us to assess whether increased Dilp2 is being released into the hemolymph of *dfmr1* mutants.

Our initial experiments specifically examined insulin signaling in the brains of *dfmr1* mutants, but to obtain a full picture of insulin signaling in *dfmr1* mutants, we need to determine if signaling is increased outside the brain. To address this question, we can examine insulin signaling in the fat body, a nutrient-sensing tissue often used to assess peripheral insulin signaling. Using both the GFP-PH reporter and staining for p-S505-Akt we can assess whether insulin signaling in the peripheral fat body mimics insulin signaling in the brain. If we found that insulin signaling was increased in the fat body, it would suggest that insulin signaling throughout the *dfmr1* mutant adult fly is increased. If, on the other hand, we found that insulin signaling was normal or decreased in the fat body, it would suggest that the brain and the periphery were experiencing different insulin signaling environments, and that some mechanism was allowing release of insulin into the brain, but perhaps not into the rest of the fly body. This experiment would gives us a better idea how extensive insulin misregulation is in the *dfmr1* mutant fly.

In this chapter, we observed that both PI3K and Akt activity were increased in the brains of *dfmr1* mutants. Interestingly, we were able to rescue the increased p-S505-Akt levels by expressing *dfmr1* in the IPCs, indicating that expression of *dfmr1* in these neurons has cell non-autonomous effects on insulin signaling. This finding is important because it shows that the increase in insulin signaling in *dfmr1* mutants likely arises due to the increased Dilp2 build-up and release from the IPCs and not due to a separate insulin signaling defect in the brain. We would have liked to confirm this finding by examining the effect of expressing *dfmr1* in the IPCs on Dilp2

levels, however we were unable to obtain more of the antibody used for the original experiments. The aforementioned tagged-Dilp2 stock may allow us to address this question so we can strengthen the finding that loss of *dfmr1* expression in the IPCs results in a cell non-autonomous increase in insulin signaling in the brain.

A final question that arises from these findings is whether insulin signaling from the IPCs is the sole factor affected by loss of *dfmr1* expression in this group of neurons. Since the IPCs are part of a larger group of neurosecretory cells (NASSEL *et al.* 2013), it seems likely that they are responsible for the production and release of neuropeptides other than Dilps. If insulin production and release are dysregulated in the IPCs, it is possible that all neuropeptides produced by these cells suffer from misregulation. It would therefore be interesting to investigate whether abnormal release of these neuropeptides contributes to phenotypes of the *dfmr1* mutant fly. However, until more is known about functions of the IPCs outside of insulin production, we cannot fully address this question. Nonetheless, we can begin to examine the contribution of the IPCs to behavior by manipulating activation of these neurons. We can perform this experiment by using transgenes that either depolarize or hyperpolarize the IPCs in *dfmr1* mutants and see what effect these manipulations have on behavior. If the IPCs are releasing excessive amounts of Dilp and are thus excessively active, we would expect that depolarizing the IPCs would either exacerbate the phenotypes of *dfmr1* mutants, or have no effect if the IPCs are already maximally depolarized. Conversely, hyperpolarization would be expected to ameliorate the *dfmr1* mutant phenotypes. To test the idea that the IPCs are hyperactive in *dfmr1* mutants, and that this contributes to the *dfmr1* phenotype, we could depolarize the IPCs in wild-type flies and see if these flies have behavioral deficits similar to *dfmr1* mutants.

Altogether, our findings bring to light another pathway misregulated in an FXS model, and reveal a group of neurons involved in circadian behavior and memory. Further experiments will answer the remaining questions, but the chief question, whether the increase in insulin signaling contributes to the behavioral deficits of the *dfmr1* mutant, will be answered in the next chapter.

Figures:

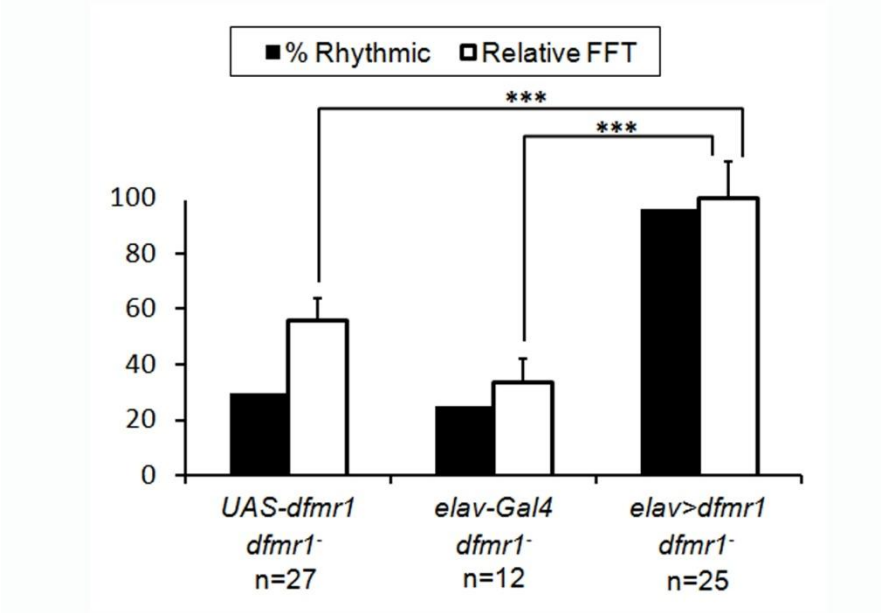

Figure 2-1. Pan-neuronal *dfmr1* expression rescues rhythmicity. Panels show the percent rhythmic (black) and relative FFT values (white) for genetic combinations testing the spatial requirement of dFMR1 expression in *dfmr1* mutants for normal circadian behavior. Relative FFT represents how the average FFT of the depicted genotype compares to the average FFT of a wild-type control. *Dfmr1* mutants expressing *dfmr1* pan-neuronally display an increased percentage of rhythmic flies and more robust circadian rhythmicity than *dfmr1* mutants with either transgene alone, $p<0.001$. Rhythmicity was determined by FFT (.01 or higher) and visual inspection of the actogram and periodogram. Statistically significant levels of rescue are denoted with asterisks (*$p<0.05$, ** $p<0.01$, ***$p<0.001$). Statistical significance was determined by a Kruskal-Wallis test and Dunn's post test. Error bars represent s.e.m. These data were collected by Xiangzhong Zheng.

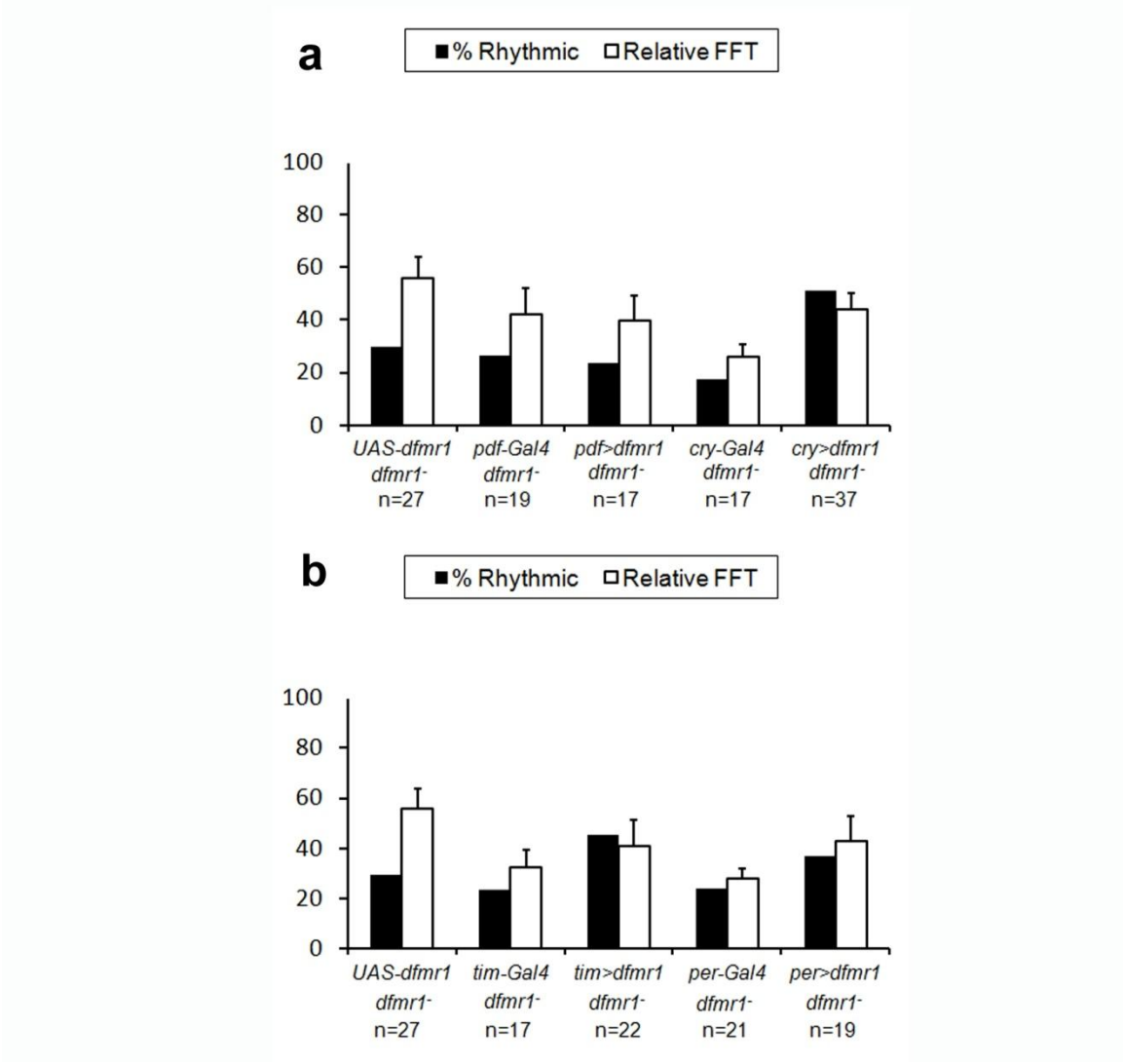

Figure 2-2. Expression of *dfmr1* in clock cells does not rescue arrhythmicity. Panels show the percent rhythmic (black) and relative FFT values (white) for genetic combinations testing the spatial requirement of dFMR1 expression in *dfmr1* mutants for normal circadian behavior. Relative FFT represents how the average FFT of the depicted genotype compares to the average FFT of a wild-type control. **(a)** Circadian behavior of *dfmr1* mutants with both *pdf-Gal4* or *cry-Gal4* and *UAS-dfmr1* is not significantly different from *dfmr1* mutants with any of the relevant transgenes alone. **(b)** Circadian behavior of *dfmr1* mutants with *tim-Gal4* or *per-Gal4* and *UAS-dfmr1* is not significantly improved compared to *dfmr1* mutants with any of the relevant transgenes alone. Rhythmicity was determined by FFT (.01 or higher) and visual inspection of the actogram and periodogram. Statistically significant levels of rescue are denoted with asterisks (*p<0.05, ** p<0.01, ***p<0.001). Statistical significance was determined by a Kruskal-wallis test and Dunn's post test. Error bars represent s.e.m. These data were collected by Xiangzhong Zheng.

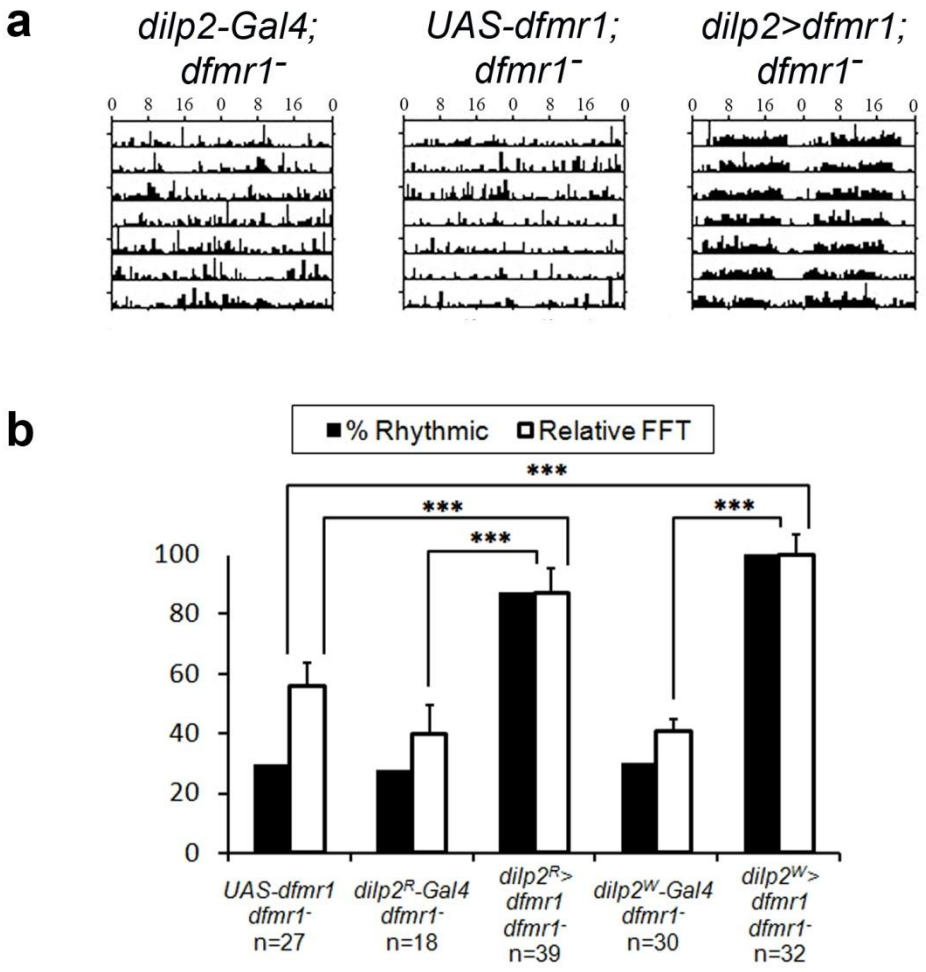

Figure 2-3. Expression of *dfmr1* in the IPCs rescues circadian rhythmicity. Panels show the percent rhythmic (black) and relative FFT values (white) for genetic combinations testing the spatial requirement of dFMR1 expression in *dfmr1* mutants for normal circadian behavior. Relative FFT represents how the average FFT of the depicted genotype compares to the average FFT of a wild-type control. **(a)** Representative actograms reveal arrhythmic free-running rest:activity patterns in *dfmr1* mutants containing either the *dilp2-Gal4* or *UAS-dfmr1* transgenes alone and normal rhythmic behavior in mutants containing both transgenes (*dilp2>dfmr1*). **(b)** Circadian behavior of *dfmr1* mutants with both $dilp2^{R}$*-Gal4* or $dilp2^{W}$*-Gal4* and *UAS-dfmr1*is significantly improved relative to *dfmr1* mutants with any of the relevant transgenes alone, $p<0.001$. Rhythmicity was determined by FFT (.01 or higher) and visual inspection of the actogram and periodogram. Statistically significant levels of rescue are denoted with asterisks (*$p<0.05$, ** $p<0.01$, ***$p<0.001$). Statistical significance was determined by a Kruskal-wallis test and Dunn's post test. Error bars represent s.e.m. These data were collected by Xiangzhong Zheng.

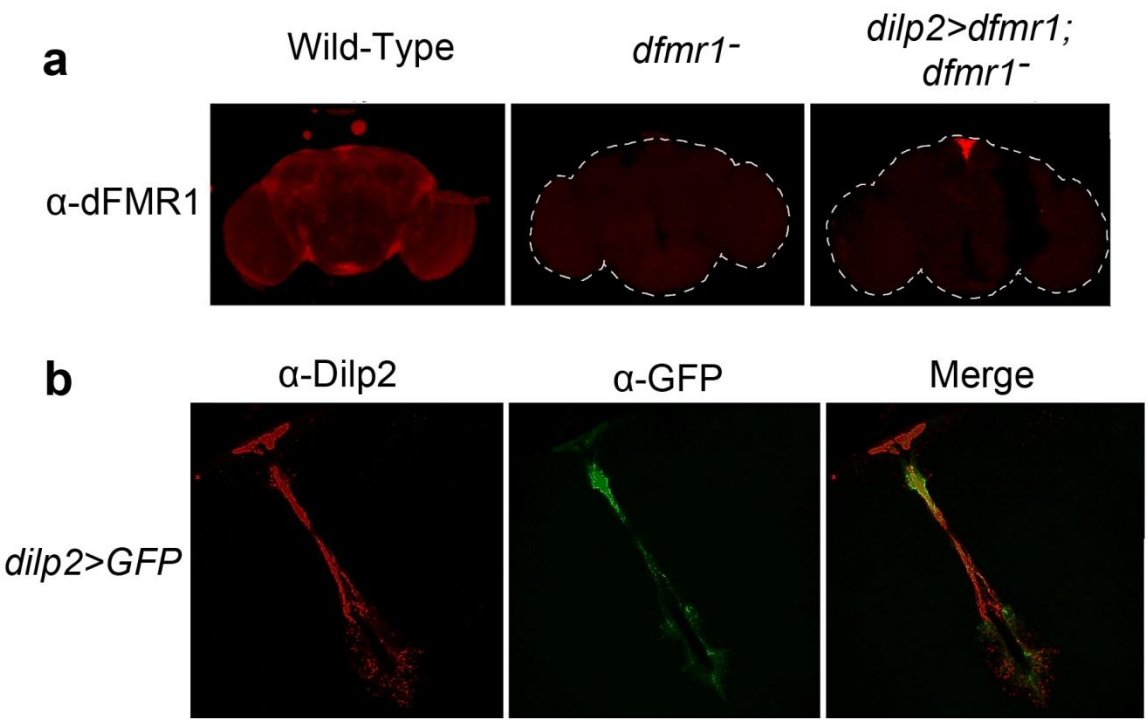

Figure 2-4. The *dilp2-Gal4* driver selectively directs expression to the IPCs. (a)Whole brains stained with anti-dFMR1 antibody show the wild type expression pattern of dFMR1 protein revealed with anti-dFMR1 staining**,** the absence of staining in a *dfmr1* null and the expression of dFMR1 protein in a null mutant brain containing both the *dilp2-Gal4* and *UAS-dfmr1* transgenes. **(b)** A control brain containing both the *dilp2-Gal4* and *UAS-CD8-GFP* transgenes immunostained to reveal Dilp2 protein expression (red), and CD8-GFP (membrane tethered) (green). The merged images demonstrate that the *dilp2-Gal4* driver directs expression to the IPCs. These data were collected by Danielle Emerson.

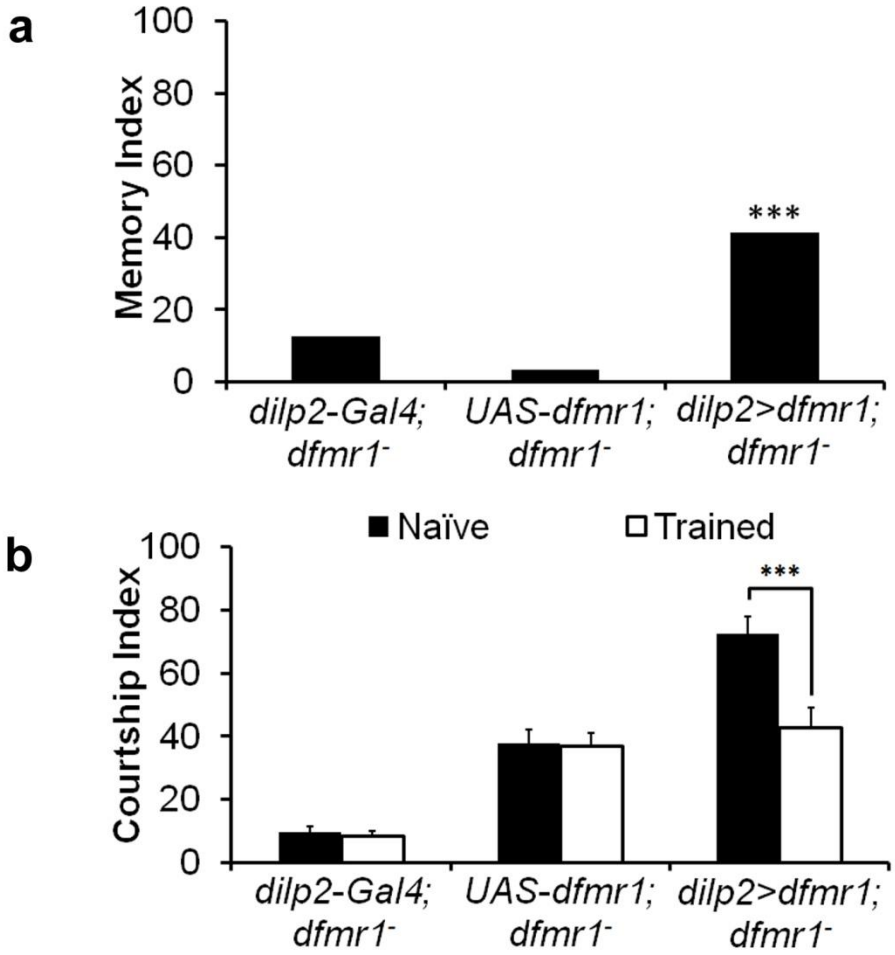

Figure 2-5. Expression of *dfmr1* in the IPCs rescues STM in the conditioned courtship paradigm. STM in the courtship paradigm is presented as **(a)** memory index (MI), which conveys the difference between the courtship indices (CIs) of trained and untrained flies in a single value ($MI = (CI_{naive}-CI_{trained})/CI_{naive}$), and **(b)** as separate CIs. If memory is present, the CI of trained flies will be reduced compared to the CI of naïve flies indicating that the trained fly remembers that negative experience of rejection by an unreceptive female. Expression of *dfmr1* in the IPCs of *dfmr1* mutants rescues STM, $p<0.0001$, N=16-20. Data were subject to an arcsine transformation to obtain a normal distribution, then statistical significance was determined by ANOVA with pre-planned comparisons. Statistically significant levels of rescue are denoted with asterisks (*$p<0.05$, ** $p<0.01$, ***$p<0.001$). Graphs depict mean ± s.e.m. These data were collected by Brian Schoenfeld and Sean McBride.

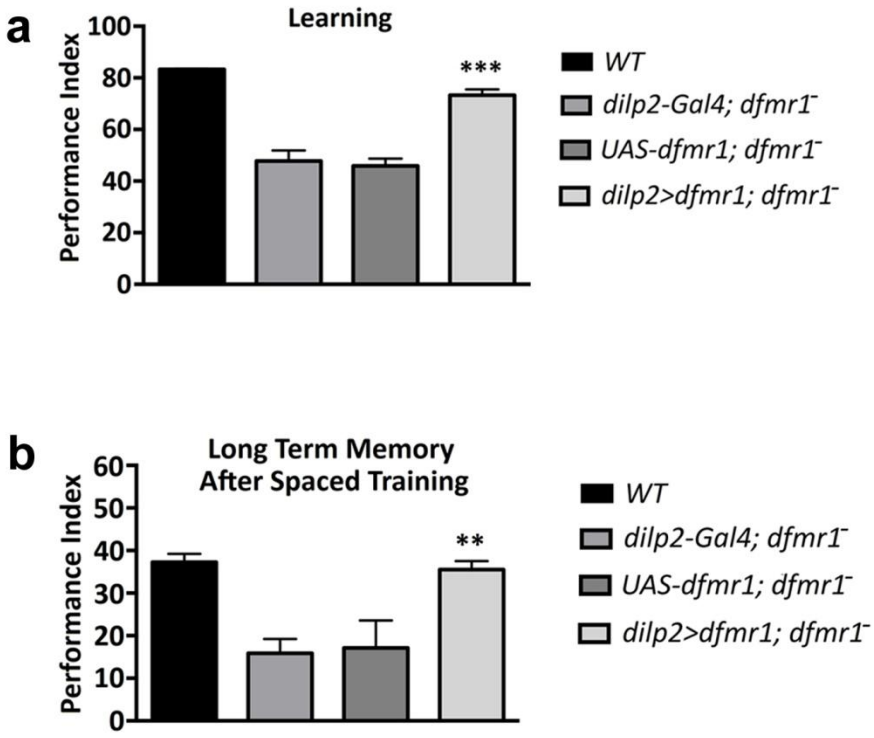

Figure 2-6. Expression of *dfmr1* in the IPCs restores learning and LTM in the olfactory conditioning paradigm. Performance index (PI) represents the percent of flies which avoid the shock-conditioned odor. (PI = % of flies avoiding shock-conditioned odor - % of flies moving toward shock conditioned odor). *Dfmr1* mutants expressing dFMR1 within the IPCs show rescue of **(a)** learning (N=4, p=0.0016), and **(b)** protein synthesis-dependent memory (N=8, p=0.0002). Statistical significance was determined by an ANOVA with Bonferroni correction and a Tukey post-test. Statistical significance is denoted with asterisks (*p<0.05, ** p<0.01, ***p<0.001). Graphs depict mean ± s.e.m. These data were collected by Daniel Chambers and Francois Bolduc.

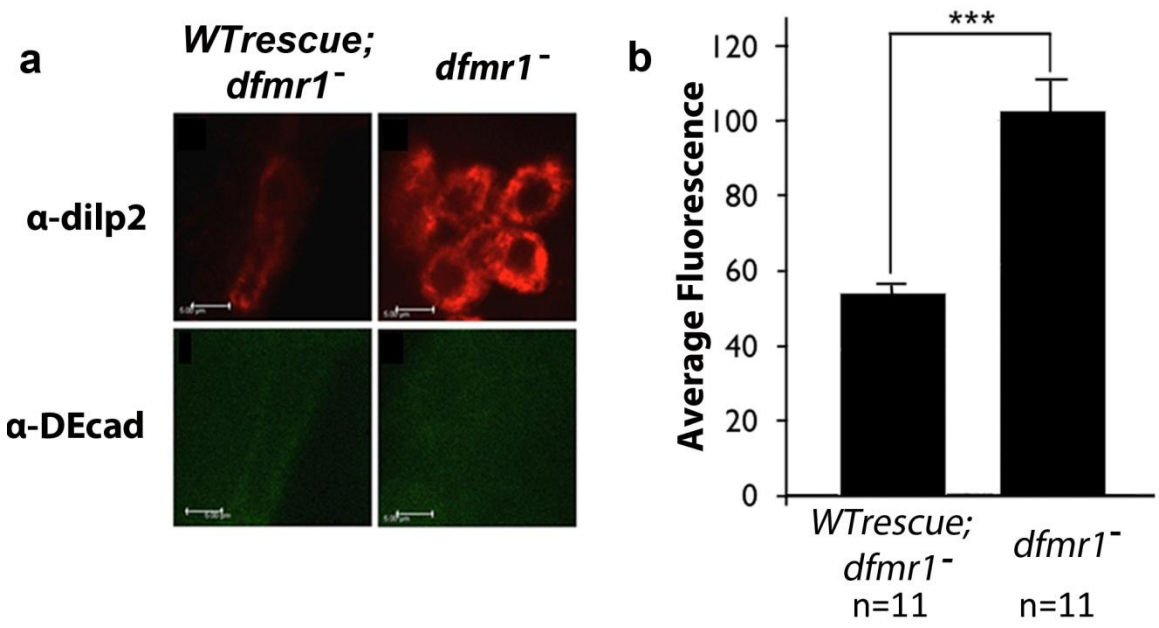

Figure 2-7. Dilp2 levels are increased in the cell bodies of the IPCs. Brains were immunostained to examine Dilp2 protein levels. **(a)** Dilp2 protein levels in the IPC cell bodies of *dfmr1* mutant brains are higher than in controls (*dfmr1* mutants containing a *WTrescue* transgene which expresses *dfmr1* at wild-type levels). DE-cadherin was used as a staining control. **(b)** Quantification reveals Dilp2 is significantly increased in *dfmr1* mutants, $p<0.001$. Statistical significance was determined by a t-test with Welch's correction. Statistical significance is denoted with asterisks (*$p<0.05$, ** $p<0.01$, ***$p<0.001$). Graphs depict mean ± s.e.m. All images in this figure are representative of quantification. These data were collected by Danielle Emerson.

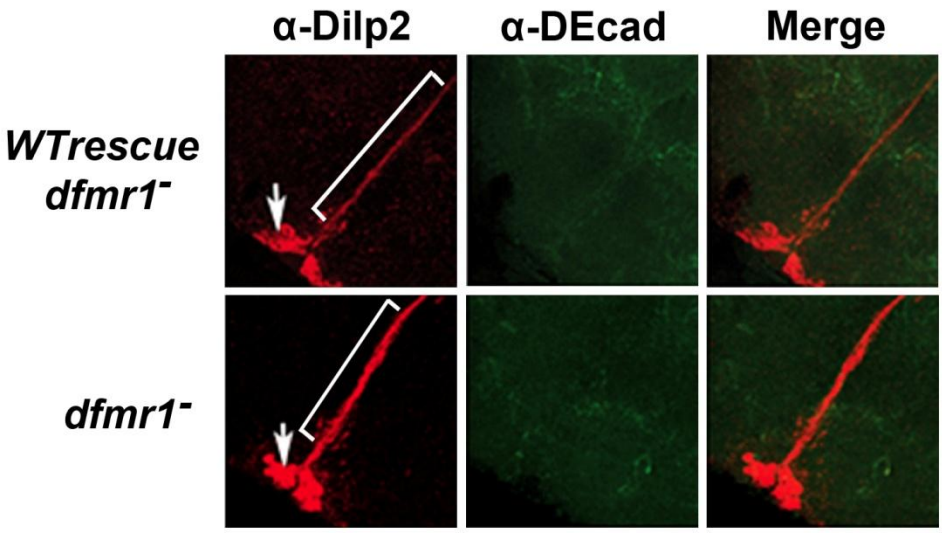

Figure 2-8. Dilp2 levels are elevated in the axons of *dfmr1* mutant IPCs. Brains from *dfmr1* mutants were immunostained with anti-Dilp2 to examine Dilp2 levels, and with anti-DE cadherin as a staining control. Increased levels of Dilp2 protein are observed in the *dfmr1* mutant IPCs relative to *WTrescue* IPCs throughout the neurites and in the cell bodies (arrowheads). Note that the gain on the confocal was increased to visualize Dilp2 staining in the axons, resulting in saturation of the IPC cell bodies in the *dfmr1* mutant brains. These data were collected by Danielle Emerson.

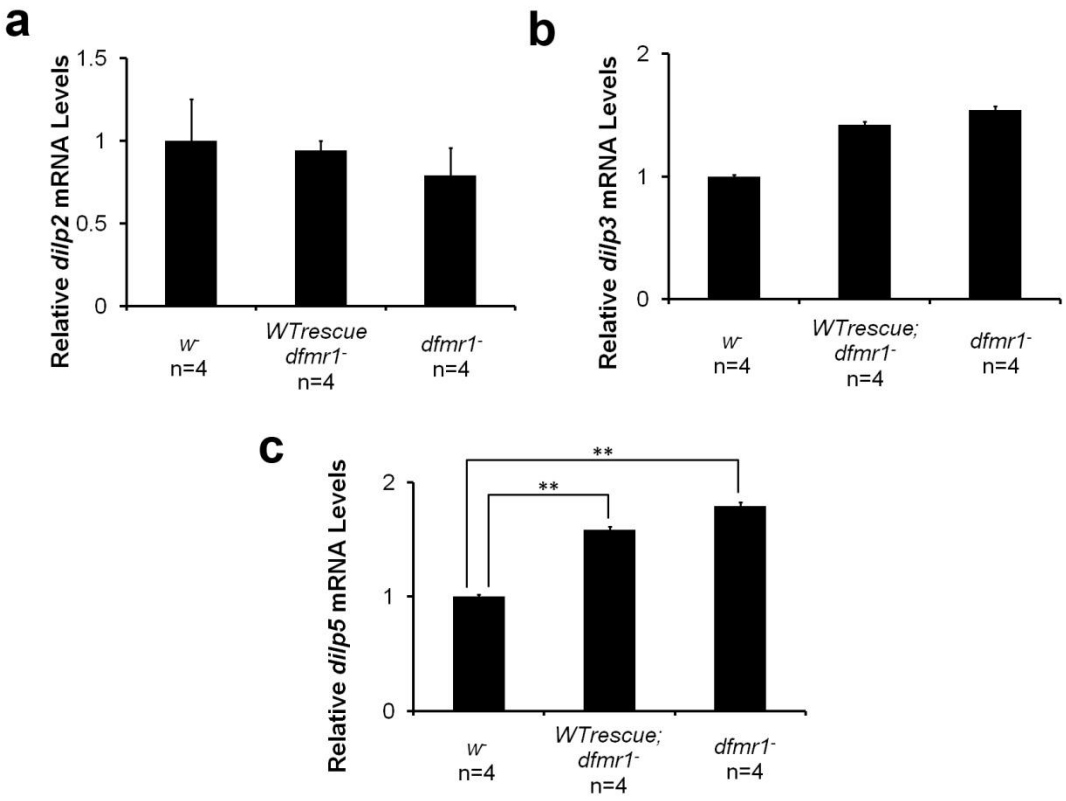

Figure 2-9. *Dilp* mRNA levels are not significantly changed in *dfmr1* mutants in relation to controls. Quantitative PCR was performed with RNA isolated from 200 heads per sample. *Dilp* mRNA levels were normalized to the geometric mean of three reference mRNAs: *SdhA, αTub84B* and *14-3-3ε*. These normalized values were then divided by the normalized value of the control genotype to determine relative mRNA expression. **(a)** *dilp2* transcript levels are not significantly changed in *dfmr1* mutants relative to w^- or *WTrescue* controls. **(b)** *dilp3* transcript levels are not significantly changed in *dfmr1* mutants relative to w^- or *WTrescue* controls. **(c)** *dilp5* transcript levels are significantly changed in *dfmr1* mutants relative to w^- controls but not compared to *WTrescue* controls. Statistical significance was determined by a Kruskal-Wallis test with a Dunn's post test. Statistical significance is denoted with asterisks (*p<0.05, ** p<0.01, ***p<0.001). Graphs depict mean ± s.e.m.

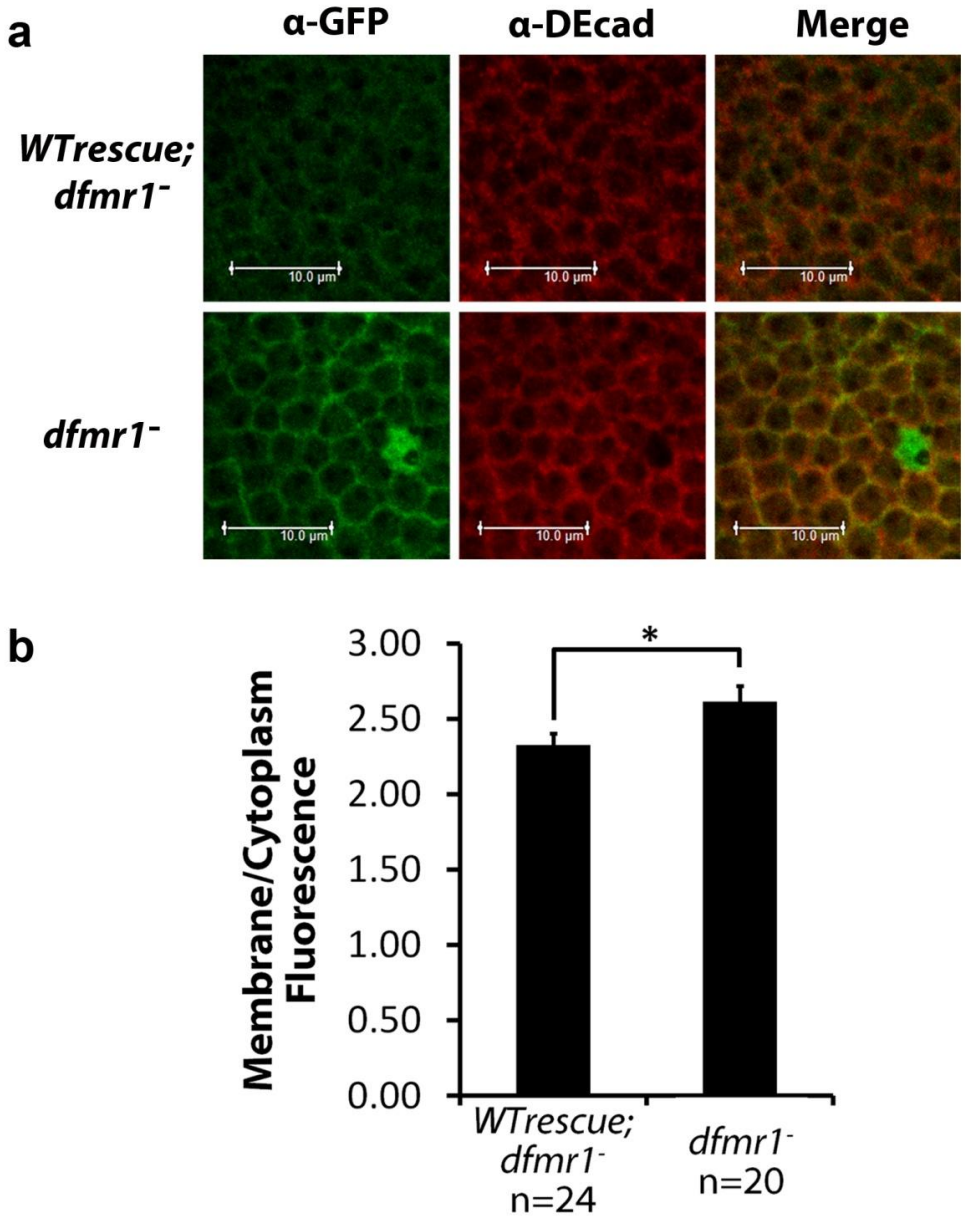

Figure 2-10. PI3K activity is increased in *dfmr1* mutant flies. (a) The GFP-PH reporter is more localized to the membrane in the cells of *dfmr1* mutant than in controls. Brains were imaged on their posterior side in the mushroom body calyx region. **(b)** Quantification of reporter distribution shown as a ratio of membrane/cytoplasm fluorescence. *Dfmr1* mutants show a significantly higher ratio, $p<0.05$. The membrane/fluorescence ratio was also calculated for the DE-cadherin staining control and was found to be the same in both genotypes (not shown). Statistical significance was determined by a Kruskal-Wallis test with a Dunn's post test. Statistical significance is denoted with asterisks (*$p<0.05$, ** $p<0.01$, ***$p<0.001$). Graphs depict mean ± s.e.m. All images in this figure are representative of quantification.

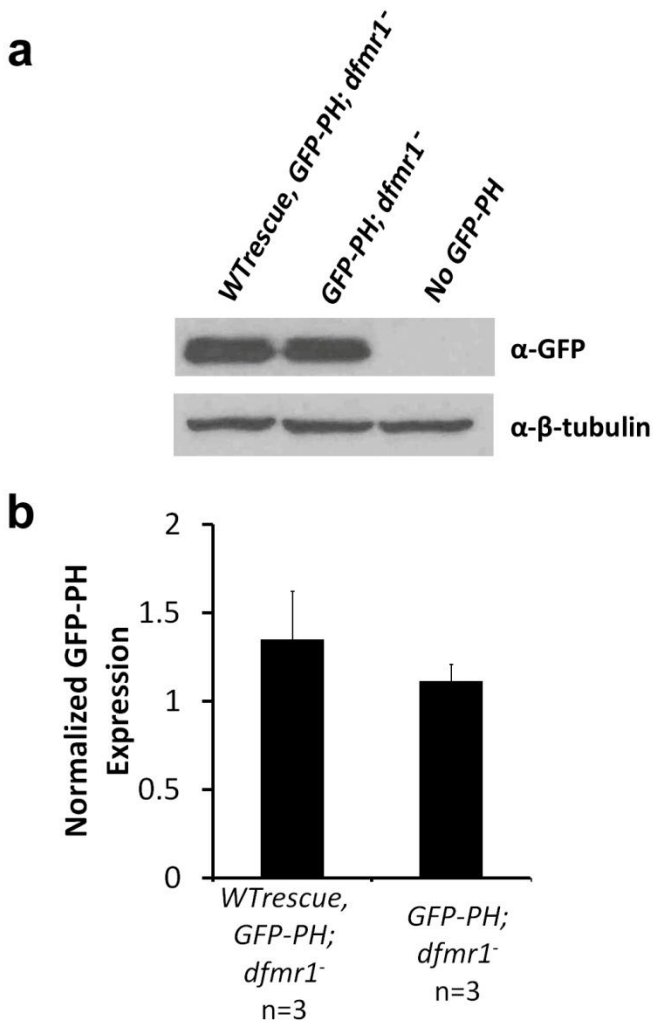

Figure 2-11. GFP-PH expression is not altered in *dfmr1* mutants. Protein was extracted from heads of *dfmr1* mutant and control flies. **(a)** A representative Western blot of head extracts probed with anti-GFP. No difference in GFP-PH levels is seen between genotypes. Extracts from a fly line not containing the GFP-PH reporter show that the band recognized by α-GFP is specific to the reporter protein. **(b)** Quantification reveals no change in GFP-PH levels between genotypes. Reporter protein levels were normalized to β-Tubulin. Statistical significance was assessed using an unpaired t-test followed by a Welch's correction.

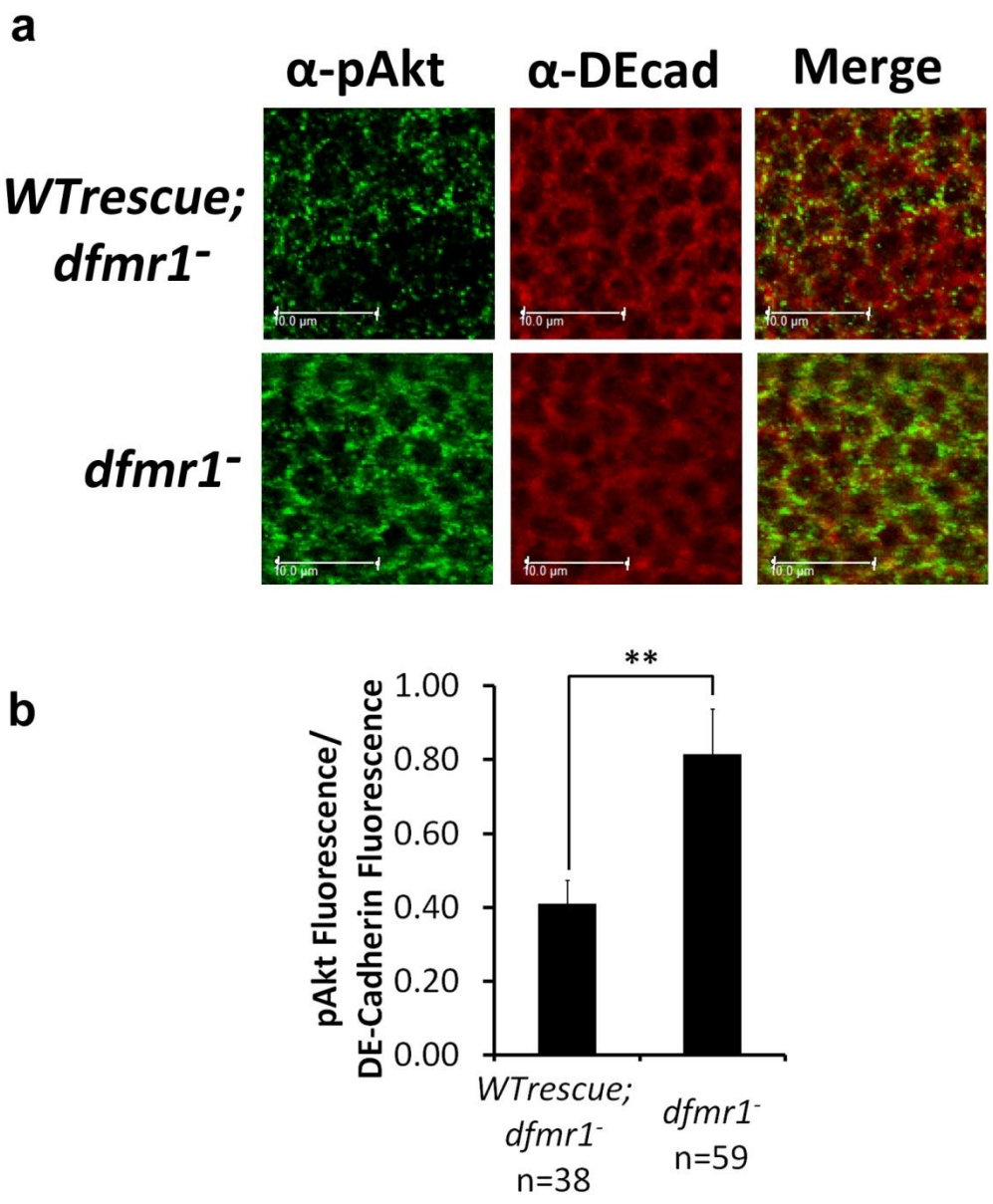

Figure 2-12. p-S505-Akt levels are increased in *dfmr1* mutants. Brains were imaged on the posterior side in the Kenyon cell region. **(a)** Whole mount brain immunostaining with anti-p-S505-Akt and anti-DEcadherin reveals that *dfmr1* mutant brains show increased p-S505-Akt levels compared to control *WTrescue* flies. **(b)** Quantification of p-S505-Akt fluorescence indicates that p-S505-Akt levels are significantly increased in *dfmr1* mutants. p-S505-Akt fluorescence was normalized to DE-cadherin. Statistical significance was determined by a Kruskal-Wallis test with a Dunn's post test. Statistical significance is denoted with asterisks (*p<0.05, ** p<0.01, ***p<0.001). Graphs depict mean ± s.e.m. All images shown are representative of quantification.

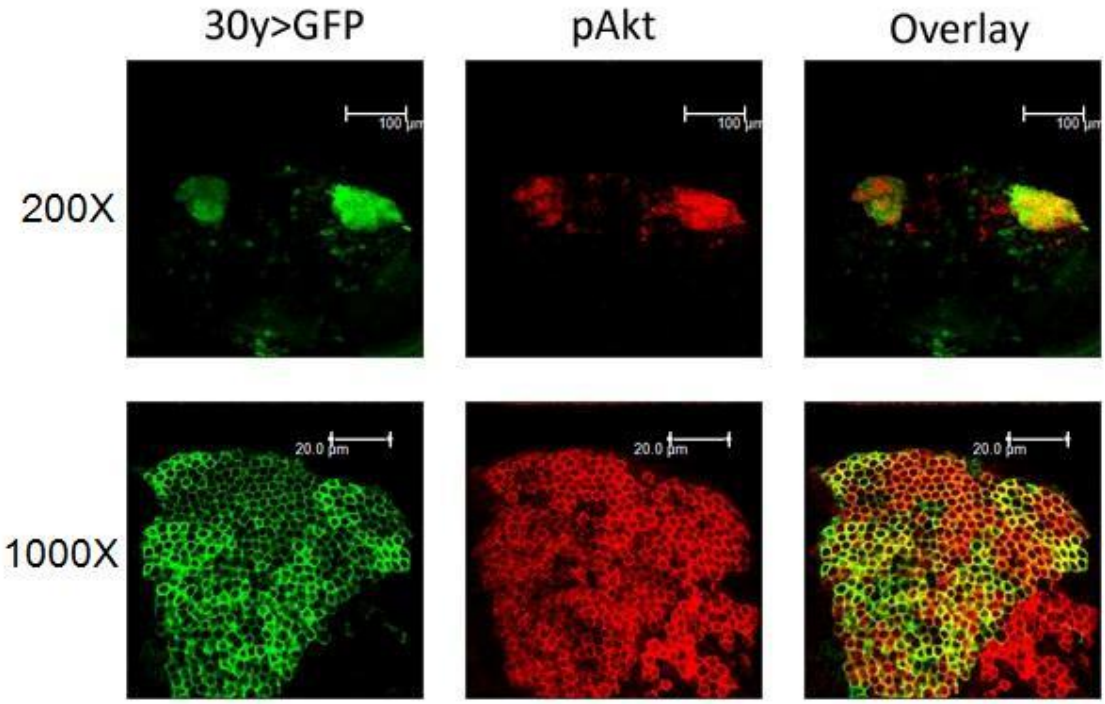

Figure 2-13. p-S505-Akt staining is almost entirely restricted to the Kenyon cells and a neighboring population of cells. Brains expressing GFP in the Kenyon cells with the *30y-Gal4* driver were stained with α-p-S505-Akt, and were imaged at both 200X and 1000X. Imaging reveals that the Kenyon cells are completely stained with p-S505-Akt, and that a small population of p-S505-Akt positive cells (red) do not overlap with the Kenyon cells (green). These images are representative of approximately 15 brains examined.

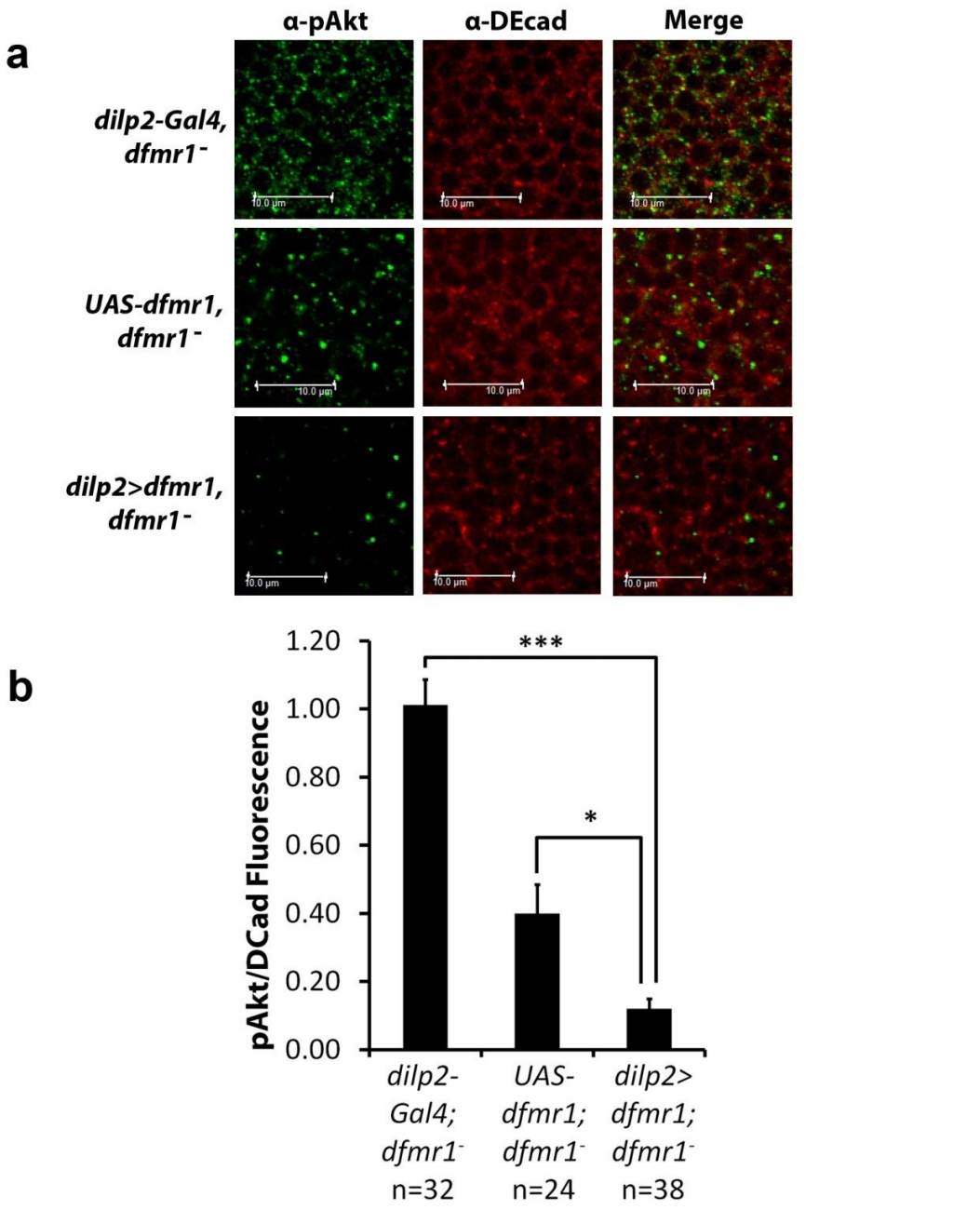

Figure 2-14. Expression of *dfmr1* in the IPCs ameliorates elevated p-S505-Akt levels in *dfmr1* mutants. (a) A notable decrease in p-S505-Akt levels is observed in *dfmr1* mutants that have *dfmr1* expressed in the IPCs. **(b)** Quantification of p-S505-Akt fluorescence reveals that *dfmr1* mutants expressing *dfmr1* in the IPCs show significantly lower p-S505-Akt fluorescence than either *dfmr1* mutant control with the driver or UAS-construct alone, $p<0.001$ and $p<0.05$. p-S505-Akt fluorescence was normalized to DE-cadherin fluorescence. Statistical significance was determined by a Kruskal-Wallis test with a Dunn's post test. Statistical significance is denoted with asterisks (*$p<0.05$, ** $p<0.01$, ***$p<0.001$). Graphs depict mean ± s.e.m. All images in this figure are representative of quantification.

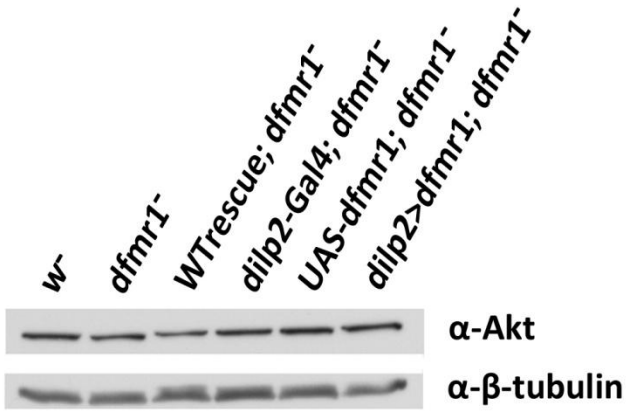

Figure 2-15. Total Akt levels are not altered in *dfmr1* mutants. Westerns performed with head extracts were probed with antibodies specific to total Akt and β-tubulin. No difference in total Akt levels is present between genotypes. These data were collected by Danielle Emerson.

Materials and Methods:

Fly stocks and maintenance

Fly stocks were maintained on standard cornmeal-molasses medium. Stocks containing the *per-Gal4* (line 2), *tim-Gal4* (line 82), *clk8.0-Gal4*, *cry-Gal4*, *pdf-Gal4* are previously described (RENN *et al.* 1999; EMERY *et al.* 2000; KANEKO AND HALL 2000; GLOSSOP *et al.* 2003). Note that we were unable to test circadian behavior of stocks containing *clk8.0-Gal4* due to lethality of this driver in combination with *UAS-dfmr1*. *Dilp2^{R}-Gal4* and *dilp2^{W}-Gal4*-containing stocks were obtained from Eric Rulifson and Peng Shen (RULIFSON *et al.* 2002; WU *et al.* 2005). The *elav-Gal4* transgene was derived from Bloomington Stock number 8765.The *dfmr1^{3}*allele and *WTrescue* are previously described in (DOCKENDORFF *et al.* 2002). Flies used for the olfactory learning assay were outcrossed into the *w1118(isoCJ1)* background.

Circadian Behavior Assay

Flies intended for circadian rhythmicity analysis were raised at 25°C on a light:dark (L:D) cycle. Male flies were collected at 0-3 days of age and maintained on standard fly food in an L:D incubator for entrainment. After 3-5 days, individual flies were loaded into 2% agar, 5% sucrose tubes, which were subsequently placed in activity monitors (TriKinetics) and maintained in dark:dark conditions for 10 days.

Data were collected in 5 minute bins and analyzed with Clock Lab software (Actimetrics) to obtain period and rhythmicity values. Rhythmicity was determined by fast fourier transform (FFT) analysis (with rhythmicity defined as a FFT value of 0.01 or more) as well as visual inspection of the actogram and periodogram. Significant differences in average FFT values between genotypes were determined using a Kruskal-Wallis test followed by a Dunn's post-test (GraphPad, InStat). Relative FFT was calculating by dividing the average FFT value of the depicted genotype by the average FFT value of the wild-type control: Relative FFT = $FFT_{depicted}/FFT_{wild\text{-}type}*100$.

Conditioned Courtship Assay

Virgin male flies were collected under CO_2 anesthesia every four hours and maintained on standard fly food in small all-male groups at 25°C in L:D until testing. Virgin X^X, *yf* test females were collected on the day of eclosion and kept in food vials. Flies were aged in a 12hr:12hr L:D incubator before behavioral training and testing was performed during the relative light phase. All male subjects were transferred to fresh control food the day before testing and assigned to random groups for behavioral training and testing. All training and testing was performed blind to genotype and treatment. A courtship index (CI) was calculated following testing as the percentage of total observation time spent courting. CIs of tested males were subjected to arcsin square root transformations to approximate normal distributions. ANOVAs were performed on pre-planned pair-wise comparisons of arcsin transformed data to get critical p-values.

For data not normal after transformation, the Mann-Whitney test was used to generate p-values (SIEGEL 1957). Statistics were performed using Statview 3.0 and Prism. Memory Index (MI) = $(CI_{naive}-CI_{trained})/CI_{naive}$ (KELEMAN *et al.* 2007).

Pavlovian olfactory learning and memory

Flies were raised at 22^{o}C and placed at 25^{o}C overnight prior to behavioral experiments. Adult *Drosophila* 1-3 days old were trained and tested with the classical conditioning procedure.

About 100 flies were trapped inside a training chamber covered with an electrifiable copper grid. Flies were allowed 90 seconds to acclimate and then were exposed sequentially to two odors, 3-octanol (OCT) and 4- methylcyclohexanol (MCH), carried through the chamber in a current of air. Relative concentrations of OCT and MCH were adjusted so that naïve flies distributed themselves 50:50 in the T-maze. Flies first were exposed for 60 seconds to the conditioned stimulus (CS+; either OCT or MCH, depending on the odor the flies were shocked to in the first step), during which time they received the unconditioned stimulus (US; twelve 1.25 seconds pulses of 60V DC electric shock at 5 second interpulse intervals). After the CS+

presentation, the chamber was flushed with fresh air for 45 seconds. Then flies were exposed for 60 seconds to a second, control stimulus (CS-; either MCH or OCT), which was not paired with electric shock. After the CS- presentation, the chamber was again flushed with fresh air for 45 seconds.

To test for conditioned odor avoidance after classical conditioning, flies were moved to the choice point of the T-maze. Ninety seconds later, the flies were exposed to two converging current of air one carrying OCT, the other MCH, from opposite arms of the T-maze. Flies were allowed to choose between the CS+ and CS- for 120 seconds, at which time they were trapped inside their respective arms of the T-maze, anesthetized and counted.

For long-term memory testing, 1-3 days old adult flies were subjected to classical (Pavlovian) olfactory conditioning for 10 training sessions without a rest interval (massed training) or to 10 training sessions with 15 minutes rest between each training session (spaced training). After training, flies were stored at 18^{o}C and then conditioned responses were tested after a 24-hour retention interval at 25°C.

For both learning and long-term memory, two groups of flies were trained and tested in one complete experiment. The CS+ was OCT and the CS- was MCH for one group; the CS+ was MCH and the CS- was OCT for the second group. The performance index (PI) was calculated as the average of the fraction of the population avoiding the shock-associated odor minus the fraction avoiding the control odor for each group of flies trained in one experiment. In other words, the PI enumerates the distribution of flies in the T-maze as a normalized “percent correctly avoiding the shock-paired odor” and ranges from 0 for a 50:50 distribution to 100 for a 100:0 distribution.

Data from an experiment were subjected to a one-way ANOVA (JMP from SAS, Inc.), followed by planned pair-wise comparisons. An alpha = 0.05 was corrected for multiple comparisons using Bonferroni. Post-test analysis was performed with the Tukey test.

Immunofluorescence

Male flies aged 3-7 days were collected for dissection. Flies were anesthetized with CO_2, then killed in 100% ethanol for 1 minute. Adult brains were dissected in 1X PBS, then placed in a siliconized microfuge tube containing 1X PBS on ice. Once dissections were complete, brains were fixed in 4% PFA for 25 minutes, washed four times in 1X PBT (0.3% Triton-X) for 10 minutes, blocked in 5% Normal Goat Serum diluted in 1X PBT, then placed in the primary antibody solution diluted in 5% Normal Goat Serum overnight at 4°C. The following day, brains were washed four times in 1X PBT and placed in secondary antibody for 3 hours at room temperature, washed four times, then mounted in a 1:5 mixture of Prolong Gold (Invitrogen, P36930) and glycerol with 2% N-propyl gallate (Sigma, P3130).

Primary antibodies used were: anti-dFMR1 (6A15) (Abcam, ab10299) 1:2000, anti-DE-cadherin (Developmental Studies Hybridoma Bank, DCAD2) 1:50, anti-GFP (Aves Labs, GFP-1020) 1:1500, anti-p-S473-Akt (D9E) (Cell Signaling Technologies, 4060) 1:800 (note that this antibody recognizes the S505 phosphorylation site in *Drosophila* which is analogous to the S473 phosphorylation site in mammals (PINAL *et al.* 2006))**,** and anti-Dilp2, a gift from Eric Rulifson.

Secondary antibodies used were: Fluorescein-conjugated donkey anti-rabbit IgG (H+L) (Jackson ImmunoResearch, 711-095-152) 1:250, (Alexa 488-conjugated donkey anti-rabbit IgG (H+L) (Life Technologies, A21206) 1:10,000, Texas Red-conjugated goat anti-mouse IgG (H+L) (Jackson ImmunoResearch, 115-075-146) 1:250, Alexa 633-conjugated goat anti-rat IgG (H+L) (Life Technologies, A21094) 1:250, and fluorescein-conjugated goat anti-chicken IgY (Aves Labs, F-1005) 1:250.

Images were taken using a Leica TCS SP microscope with the settings kept constant throughout imaging. Leica LAS AF Lite software was used for quantification of confocal images. Significance was determined using an unpaired t-test with Welch's correction or Mann-Whitney test (GraphPad, InStat).

For quantification of Dilp2 staining, three separate experiments were performed with 12 brains imaged for each genotype. For each brain, a z-stack was taken of the IPCs, and individual

images were used for quantification. Five cells were chosen for each brain and the average pixel intensity was recorded for 6 elliptical areas in the cell cytoplasm.

When imaging GFP-PH stained brains, images were taken with the 100X objective and 2X zoom of the posterior surface of the brain in the mushroom body calyx region because this area contains a large number of easily imaged cell bodies. Laser and other microscope settings were the same for all brains imaged. Brains were stained with anti-GFP to visualize the GFP-PH reporter, and with anti-DEcadherin to mark the plasma membrane. Leica LAS AF Lite software was used to measure the average ratio of membrane fluorescence/cytoplasm fluorescence for 20 cells in each hemisphere of the brain. These numbers were used to obtain the average membrane/cytoplasm ratio for each genotype.

When quantifying pAkt staining, images of the posterior surface of the brain were taken with the 100X objective in the mushroom body calyx region. Laser and other microscope settings remained the same for all brains imaged. Brains were stained with anti-p-S505-Akt and anti-DEcadherin to visualize the plasma membrane. Leica LAS AF Lite software was used to measure the average p-S505-Akt and DE-cadherin fluorescence of the cells in region, then the fluorescence value obtained for p-S505-Akt was normalized to that of DE-cadherin to control for staining differences between brains. Both hemispheres of the brain were imaged and the normalized p-S505-Akt values for each side of the brain were averaged together. These numbers were used to compute the average normalized p-S505-Akt value for each genotype.

Western analysis

The heads of 3-7 day old flies were removed and placed into tubes on dry ice. Lysates were prepared in extraction buffer containing 20mM HEPES (pH 7.5), 100mM KCL, 5% glycerol, 100µM Na_3VO_4, 10mM EDTA, 0.1% Triton-X 100, 1mM DTT, 5mM EGTA, 1x Protease Inhibitor Cocktail (Sigma, P2714). Lysate preparation, SDS-PAGE, Western analysis and quantification were performed as previously described (PEPPER *et al.* 2009). Primary antibodies used were: Anti-β-tubulin (Developmental Studies Hybridoma Bank, E7) 1:20,000, Anti-Akt (pan) (C67E7)

(Cell Signaling Technologies, 4691) 1:5000, Anti-GFP (Aves Labs, GFP-1020) 1:2,500. Secondary antibodies used were: HRP-conjugated goat anti-mouse IgG (H+L) (Jackson ImmunoResearch, 115-055-146) 1:5000 or 1:10,000, HRP-conjugated goat anti-rabbit IgG (H+L) (Jackson ImmunoResearch, 111-055-144) 1:2000, HRP-conjugated goat anti-chicken IgY (IgG) (H+L) (Jackson ImmunoResearch 103-035-155) 1:20,000. Quantification of Western blots was done in ImageJ, with three to four biological replicates. Average band intensity of the protein of interest was normalized to a loading control (β-tubulin). Then relative intensities were calculated as the ratio of the protein of interest to wild type.

RNA isolation and quantitative PCR

Heads were homogenized in Tri-Reagent (Sigma) and RNA was isolated and purified using the RNeasy Mini Kit (Qiagen, 74104). cDNA was synthesized with Superscript III (Invitrogen, 18080-051) and Oligo dT primers. qPCR was performed using Brilliant III Ultra Fast Sybr Master Mix (Agilent Technologies, 600882) on the MxPro 3000 system (Agilent Technologies). *Dilp* transcript levels were normalized to three reference genes (*SdhA*, α*Tub84B* and *14-3-3*ε) by taking the geometric mean as described in (VANDESOMPELE *et al.* 2002) to account for potential inconsistencies in housekeeping gene expression between genotypes. All three housekeeping genes showed consistent expression between genotypes, suggesting that all are appropriate reference genes for *dfmr1*mutants. Three technical replicates and four biological replicates (200 heads/sample) were run for each condition.. *Dilp2, Dilp3* and *Dilp5* primers are described in (GRONKE *et al.* 2010) and *SdhA* primers are described in (LING AND SALVATERRA 2011).The *αTub84B* primers were: forward: 5'-CTTGTCGCGTGTGAAACACT-3' and reverse: 5'AGCAGTAGAGCTCCCAGCAG-3' and the *14-3-3*ε primers were: forward: 5'-GAGCGCGAGAACAATGTGTA-3' and reverse: 5'-ACGGTCAGCTCTACGTCCAT-3'. All primer concentrations were optimized to produce 100% amplification efficiency. Statistical significance was determined by a Kruskal-Wallis test with a Dunn's post-test.

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Chapter 3: Aberrant insulin signaling in *dfmr1* mutants contributes to circadian and memory defects

*Portions of this chapter are excerpted with modification from Monyak RE, Emerson D, Schoenfeld BP, Zheng X, Chambers DB, Rosenfelt C, Langer S, Hinchey P, Choi CH, McDonald TV, Bolduc FV, Sehgal A, McBride SM, Jongens TA. 2016. Insulin signaling misregulation underlies circadian and cognitive deficits in a *Drosophila* fragile X model. *Mol Psychiatry.* doi: 10.1038/mp2016.51 [Epub ahead of print]

Abstract:

In the previous chapter, we showed that several markers of insulin signaling are increased in the brains of *dfmr1* mutant flies. These results raise the question of whether abnormal insulin signaling affects circadian behavior and memory. To determine whether increased insulin signaling might contribute to arrhythmic circadian behavior in *dfmr1* mutants, we decreased insulin signaling genetically using four different mutations targeting the insulin signaling pathway. In all cases, reduction of insulin signaling rescued circadian behavior, indicating that normalized insulin signaling is necessary for rhythmic circadian behavior. However, increasing insulin signaling in an otherwise wild-type background did not recapitulate the arrhythmicity phenotype of *dfmr1* mutants, indicating that increased insulin signaling is not sufficient to explain arrhythmicity in *dfmr1* mutants.

We also tested whether genetically reducing insulin signaling could rescue courtship-based and olfactory-based memory deficits seen in *dfmr1* mutants. Interestingly, we found that both forms of memory were rescued by insulin signaling reduction, indicating that misregulated insulin signaling in *dfmr1* mutants also contributes to memory defects. Furthermore, we found that we could also rescue courtship-based STM and olfactory-based LTM through pharmacological insulin signaling normalization using the anti-diabetes drug metformin. These results indicate that misregulated insulin signaling has behavioral implications and that further exploration of the nature of its misregulation is warranted.

Introduction:

Fragile X Syndrome (FXS) is the most prevalent cause of intellectual disability and autism (TURK 2011). Patients with FXS also present with a high incidence of many other cognitive and behavioral abnormalities, including Attention Deficit Hyperactivity Disorder, seizures, and disturbed sleep (GARBER *et al.* 2008; KIDD *et al.* 2014). Interestingly, loss of a single gene, *Fmr1,* is responsible for the multi-faceted symptoms of this disease (DE VRIES *et al.* 1998). To understand how loss of this single gene causes behavioral dysregulation, we study *Drosophila* that have a null mutation in *dfmr1,* the sole functional homolog of *Fmr1* (WAN *et al.* 2000). These flies exhibit robust defects in circadian rhythmicity (DOCKENDORFF *et al.* 2002; INOUE *et al.* 2002; MORALES *et al.* 2002), which may help us explain the sleep defects seen in human FXS patients. The *dfmr1* null flies also show memory defects in both the olfactory conditioned paradigm and in the conditioned courtship paradigm (MCBRIDE *et al.* 2005; BOLDUC *et al.* 2008), mirroring the cognitive dysfunction frequently seen in FXS patients.

In the previous chapter, we presented evidence that expression of *dfmr1* in the insulin-producing cells (IPCs) is important for circadian rhythmicity and memory, as well as normal insulin signaling in the brain. *Dfmr1* mutants were found to have increased activity of PI3K and Akt in the mushroom body calyx region of the brain, a trait that was rescued by expression of *dfmr1* in the IPCs. Since insulin signaling is critical for neurogenesis and neural morphology, the increased insulin signaling present in *dfmr1* mutants could have detrimental effects on brain function (FERNANDEZ AND TORRES-ALEMAN 2012). A key question raised by our findings is therefore whether misregulated insulin signaling in *dfmr1* mutants causes memory or circadian defects.

The insulin signaling pathway is complicated, and overlaps with many other signaling pathways. In a simplified version of insulin signaling, *Drosophila* insulin-like peptides (Dilps) bind the insulin receptor (InR), initiating activation of phosphoinositide-3-kinase (PI3K) through phosphorylation (TELEMAN 2010). The now activated PI3K phosphorylates the membrane phospholipid phosphotidylinositol (4,5)-biphosphate (PIP2), converting it to phosphotidylinositol

(3,4,5)-triphophate (PIP3). Increased levels of PIP3 in the membrane recruit Akt, which is then activated through phosphorylation by phosphoinositide-dependent kinase-1 (PDK1). PTEN (also known as phophotase and tensin homolog deleted on chromosome 10) antagonizes PI3K activity by dephosphorylating PIP3 to convert it back to PIP2 (TELEMAN 2010). In *Drosophila,* PI3K is composed of two subunits: the DP110 catalytic subunit, and the adaptor protein p60 (LEEVERS *et al.* 1996). Deletion of one of the components of this pathway, save for the repressor PTEN or the redundant Dilps) has dramatic effects on growth (LEEVERS *et al.* 1996; BROGIOLO *et al.* 2001). Notably, the insulin signaling pathway in *Drosophila* is highly conserved with that of mammals (TELEMAN 2010).

Interestingly, though several drugs have been shown to effectively target the insulin signaling pathway, the most well-used is metformin, a old drug with an incompletely understood mechanism. Metformin, which is derived from the *Gallega officinalis* plant, has been used as a diabetes treatment for hundreds of years in plant-form (ROJAS AND GOMES 2013). Although this drug is still poorly understood, it does not exhibit many of the serious long-term side effects present in other drugs that target the insulin signaling pathway (ROJAS AND GOMES 2013). Indeed, metformin is so safe that it has been used effectively and safely to treat children showing signs of pre-diabetes (ROJAS AND GOMES 2013).

Since insulin signaling is abnormal in *dfmr1* mutants, as shown in the previous chapter, we wanted to determine whether normalization of insulin signaling would have positive effects on the behavior of these flies. An improvement in behavior would suggest that the misregulated insulin signaling pathway in *dfmr1* mutants contributed to their abnormal behavioral phenotypes.

In this chapter, we show that genetically decreasing insulin signaling using four genetic manipulations rescues circadian behavior in *dfmr1* mutants. Furthermore, we show that genetic reduction of insulin signaling is able to rescue short-term memory (STM) in the conditioned courtship paradigm, as well as learning and long-term memory (LTM) in the olfactory conditioning paradigm. These results indicate that insulin signaling contributes to circadian and cognitive defects in *dfmr1* mutant flies, and further suggest that alleviating the misregulation of insulin

signaling would have positive effects on behavior. To determine if pharmacological normalization of insulin signaling would rescue behavior we treated *dfmr1* mutants with metformin and revealed that adulthood drug treatment was sufficient to rescue defective STM in the conditioned-courtship paradigm, and to rescue learning and LTM in the olfactory-conditioning paradigm. Altogether, these results indicate that misregulated insulin signaling contributes to cognitive and circadian defects in *dfmr1* mutants, and that reducing insulin signaling effectively ameliorates these problems. Our results also identify metformin as possible therapy for cognitive difficulties in FXS.

Results:

Reducing insulin signaling rescues circadian rhythmicity in dfmr1 mutants

In Chapter 2, we demonstrated that expression of *dfmr1* specifically in the IPCs corrected insulin signaling in *dfmr1* mutants, suggesting that the circadian phenotype displayed by the mutants could be rescued by reducing signaling through this pathway. To directly test the effect of reducing insulin signaling in *dfmr1* mutants, we performed four independent genetic manipulations in the *dfmr1* mutant background. We first tested whether ubiquitous reduction of insulin signaling would improve rhythmicity by genetically reducing the gene dosage of *dilp2* and *InR* by introducing a null allele of *dilp2* or a strong hypomorphic allele of *InR* into the *dfmr1* mutant background**.** Examination of circadian activity in these flies revealed a significant increase in rhythmicity compared to *dfmr1* mutant controls (Figure 3-1a & b). We then tested whether pan-neuronal reduction of insulin signaling would rescue rhythmicity in *dfmr1* mutants. We genetically reduced PI3K activity by pan-neuronally expressing a dominant negative form of the 110kD catalytic subunit (*UAS-DP110*^{DN}) in the *dfmr1* mutant background. We also elevated the expression of the PI3K antagonist, PTEN, using the *UAS-PTEN* transgene in combination with *elav-Gal4*. These two neuron-specific genetic manipulations led to statistically significant rescue of the free-running rhythm defect displayed by *dfmr1* mutants (Figure 3-2a & b). These results confirm that elevated insulin signaling contributes to the arrhythmic circadian behavior displayed by *dfmr1* mutants.

Increasing insulin signaling in wild-type flies does not lead to circadian defects

To determine if increased insulin signaling is sufficient to induce circadian defects, we used several genetic techniques to increase insulin signaling in wild-type flies. First, we overexpressed Dilp2 in the IPCs using the UAS/Gal4 system and a *dilp2-Gal4* driver. However, this manipulation did not result in any defects in circadian behavior (Figure 3-3). Since it has previously been show that Dilp2 can accumulate in the IPCs without release (KAPLAN *et al.* 2008; GEMINARD *et al.* 2009), increasing Dilp2 level in these neurons may not have adequately increased insulin signaling in the flies. We then attempted to overexpress Dilp2 more broadly using the pan-neuronal *elav-Gal4* driver. Again, this broader overexpression of Dilp2 failed to decrease circadian rhythmicity in wild-type flies (Figure 3-3).

Since Dilps function redundantly (GRONKE *et al.* 2010), it is possible that overexpression of Dilp2 leads to compensatory reductions in the other Dilps. We then tried increasing insulin signaling by pan-neuronally expressing *UAS-InR*^{CA}, a constitutively active InR, and *UAS-myr-Akt*, a constitutively active form of Akt. However, we were unable to detect any detrimental effect on circadian behavior from these manipulations (Figure 3-4a & b). These results suggested that increasing insulin signaling might not be sufficient to recapitulate the circadian rhythmicity defects of *dfmr1* mutants. The second possibility is that insulin signaling must be increased outside the nervous system to cause arrhythmicity in wild-type flies. Unfortunately, our attempts to ubiquitously increase insulin signaling using the *tub-Gal4* driver paired with *UAS-dilp2, UAS-InR*^{CA} or *UAS-myr-Akt* resulted in lethality. Indeed, it has previously been reported that too high ubiquitous insulin signaling can be lethal (IKEYA *et al.* 2002). Altogether, our inability to reproduce circadian arrhythmicity in wild-type flies with increased insulin signaling suggests that while insulin signaling plays a role in the circadian phenotype of *dfmr1* mutants, it may not be the sole cause of this defect.

Aberrant insulin signaling contributes to memory defects in dfmr1 mutants

To directly test if reduced insulin signaling corrects the STM defects, we tested STM in *dfmr1* mutants that carried one null allele of *dilp2*, or that had pan-neuronal expression of $DP110^{DN}$ or PTEN. We were unable to test the *InR* mutation because it almost completely abolished courtship behavior. We found that STM was restored in *dfmr1* mutants by genetically reducing the gene dosage of *dilp2* (Figure 3-5a & b). Pan-neuronal expression of $DP110^{DN}$ also restored memory (Figure 3-6a & b) as did overexpression of PTEN (Figure 3-7a & b). Interestingly, pan-neuronal expression of $DP110^{DN}$ in wild-type flies eliminated STM (Figure 3-6a & b), suggesting that decreases in normal insulin signaling can negatively affect memory. Altogether, these results indicate that enhanced insulin signaling contributes to the STM defect in *dfmr1* mutants.

Since normalization of insulin signaling restored STM in *dfmr1* mutants, we explored how alteration of insulin signaling affected defective learning and LTM in the classical conditioning olfactory memory paradigm. Genetic reduction of insulin signaling by pan-neuronal expression of $DP110^{DN}$ restored learning and LTM, while *dfmr1* mutant controls with either the *elav-Gal4 or UAS-*$DP110^{DN}$ transgenes alone did not exhibit improved learning or LTM (Figure 3-8a & b). These results indicate that elevated insulin signaling also contributes to the defective olfactory-based memory seen in *dfmr1* mutant flies.

Metformin treatment ameliorates memory defects in dfmr1 mutants

Given the misregulation of insulin signaling in *dfmr1* mutants, we explored treatment with metformin, a widely-used drug for type 2 diabetes that acts as an insulin signaling sensitizer. Several mechanisms have been suggested to explain the efficacy of metformin in the treatment of type 2 diabetes, but we selected this drug because it is known to increase PTEN expression and AMPK activation, and to decrease TOR signaling (ZHOU *et al.* 2001; LEE *et al.* 2010; KIM AND CHOI 2012). We found that *dfmr1* mutant flies reared on food containing metformin for 4-6 days after

eclosion exhibited restored STM in the conditioned courtship memory paradigm in contrast to mutant flies fed food containing only vehicle (Figure 3-9a & b). We also tested classical olfactory conditioning memory in adult *dfmr1* mutant flies treated acutely with metformin overnight prior to training. We found that metformin treatment rescued both olfactory learning and protein synthesis-dependent LTM in *dfmr1* mutants (Figure 3-10a & b). We replicated previous results in which *dfmr1* mutant flies showed no defects in olfaction or shock sensitivity (BOLDUC *et al.* 2008), and also observed that metformin did not exert its effect via enhanced olfaction or shock reactivity (Figure 3-11a-c), confirming that metformin rescues cognitive rather than sensory defects. These results show that treatment with a drug known to target the insulin signaling pathway rescues several forms of memory in *dfmr1* mutant flies.

Discussion:

In this chapter, we show that misregulation of insulin signaling in *dfmr1* mutants contributes to abnormal memory and circadian rhythmicity. First, we reveal that genetically reducing insulin signaling using genetic mutations that downregulate the downstream insulin pathway rescues circadian rhythmicity, STM defects in the conditioned courtship paradigm, and LTM defects in the olfactory conditioning paradigm. However, increasing insulin signaling was not sufficient to recapitulate circadian arrhythmicity in wild-type flies. We were also able to rescue both STM and LTM by acute administration of metformin in adulthood. These results suggest that reduction of insulin signaling could be a potential therapy for FXS, and that a better understanding of how the insulin signaling pathway is involved in memory and circadian behavior is important for better understanding both these phenotypes.

We showed that genetically reducing insulin signaling rescues circadian behavior. It is interesting to note that reduction of insulin signaling worked both when it occurred ubiquitously, as with the *dilp2* and *InR* mutations, and when it occurred pan-neuronally, as with the PI3K dominant negative, $DP110^{DN}$, or by overexpression of the PI3K repressor *PTEN*. This observation shows that reduction of insulin signaling in the neurons alone is sufficient to rescue the circadian

defect, suggesting that the origin of the circadian defect lies in the nervous system. This finding suggests an experiment in which we attempt to determine in which neurons reduction of insulin signaling is sufficient to rescue circadian behavior. The results of this experiment would indicate which neurons are being overactivated by insulin signaling and affecting circadian behavior, and would help us elucidate the neural circuits affecting circadian behavior in *dfmr1* mutants.

We also found that increasing insulin signaling in a wild-type fly was not sufficient to cause arrhythmicity. This result suggests that an increase in insulin signaling alone is not enough to replicate the *dfmr1* mutant circadian phenotype, and thus that other factors must be contributing to the phenotype as well. However, it should be noted that we were unable to increase insulin signaling ubiquitously due to lethality because the *tub-Gal4* driver was too strong, an effect that has been previously noted (IKEYA *et al.* 2002). It would therefore be important to try increasing insulin signaling using the weaker *act-Gal4* driver to determine if a whole body increase in insulin signaling might be necessary to recapitulate the *dfmr1* circadian arrhythmicity phenotype. A discovery that insulin signaling needed to be increased ubiquitously would indicate that we needed to look outside the nervous system to reveal all the tissues responsible for the *dfmr1* mutant circadian phenotype.

We also found that reduction of insulin signaling using three different genetic manipulations could rescue STM in the conditioned courtship paradigm, and that reduction of PI3K activity rescued learning and LTM in the olfactory conditioning paradigm. As with circadian rhythmicity, reduction of insulin signaling in the nervous system alone was able to rescue both forms of memory, indicating that the increase in insulin signaling is likely affecting one or several groups of neurons, and that identifying these groups could be helpful in understanding the circuitry defects underlying memory. Furthermore, it would be interesting to see how much overlap exists in the circuitry that requires normalized insulin between the circuits necessary for olfactory-based memory and for courtship-based memory. We did not test whether increasing insulin signaling in wild-type flies would affect memory. However, it would be a useful experiment

to determine if insulin signaling is the sole pathway contributing to memory defects in *dfmr1* mutants.

One of the advantages of the conditioned courtship assay is that it gives us information about naïve courtship levels—a trait that is strikingly reduced in *dfmr1* mutants, indicating a defect in normal social behavior (DOCKENDORFF *et al.* 2002). Unfortunately, we were unable to assess this phenotype due to the effects of eye color on courtship level. Our wild-type background is w^{1118}, and these flies have a null mutation in the *white* gene that gives them a white-eyed phenotype. This mutation also renders them blind and thus inhibits their courtship behavior (KRSTIC *et al.* 2013). However, the transgenes we used to reduce insulin signaling contain a w^{+} marker that rescues courtship levels to varying degrees depending on the strength of *white* expression. This issue made it impossible for us to determine when rescue of naïve courtship was due to increased *white* expression, or decreased insulin signaling. To avoid confounding effects of *white* expression in the future, we can use flies in our preferred *iso31B* background that express a wild-type *white* gene crossed into that background. Although this method will slightly extend stock generation time, it will remove any confounding effects on courtship and will henceforth allow us to assess social behavior in *dfmr1* mutants.

Finally, we showed that acute metformin treatment is able to rescue both courtship-based and olfactory-based memory deficits in *dfmr1* mutant flies. This finding raises the question of the mechanism by which the rescue occurs. The most likely possibility is that metformin directly represses the insulin signaling pathway to normalize memory in *dfmr1* mutants. Indeed, metformin is known to increase AMPK activity (SLACK *et al.* 2012), which indirectly represses TOR, and would thus repress the output of insulin signaling. To determine if metformin rescues memory in *dfmr1* mutants through AMPK, we could first determine if activation of AMPK is sufficient to rescue memory by treating flies with AICAR, a drug with activates AMPK. If AICAR treatment rescues memory in *dfmr1* mutants, we would want to test whether the presences of AMPK was necessary for metformin action by testing whether metformin could still rescue memory in *dfmr1* mutant flies heterozygous for a mutation in AMPK. If AMPK is necessary for

metformin action, metformin-mediated rescue of memory should be inhibited in flies with decreased AMPK levels. Conversely, if metformin rescues memory through a pathway independent of AMPK, decreased AMPK levels should not affect memory rescue. The identification of the pathways targeted by metformin in *dfmr1* mutants would extend our understanding of memory defects in *dfmr1* mutants, and would reveal other signaling pathways that could be targeted pharmacologically. This information would be useful if metformin treatments prove effective in the mouse *Fmr1* KO and testing on human patients is considered.

In this chapter, we revealed that defective insulin signaling in *dfmr1* mutants contributes to the behavioral phenotypes exhibited by these flies. We also reveal a potential drug treatment that rescues memory defects in adulthood. This latter result hints that the memory defect is not of developmental origin, and thus that if adulthood rescue with metformin is effective in a mammalian FXS model, metformin might be valuable treatment for human patients even later in life. In the next chapter, we will further explore the developmental requirements of normalized insulin signaling for normal behavior in *Drosophila.*

Figures:

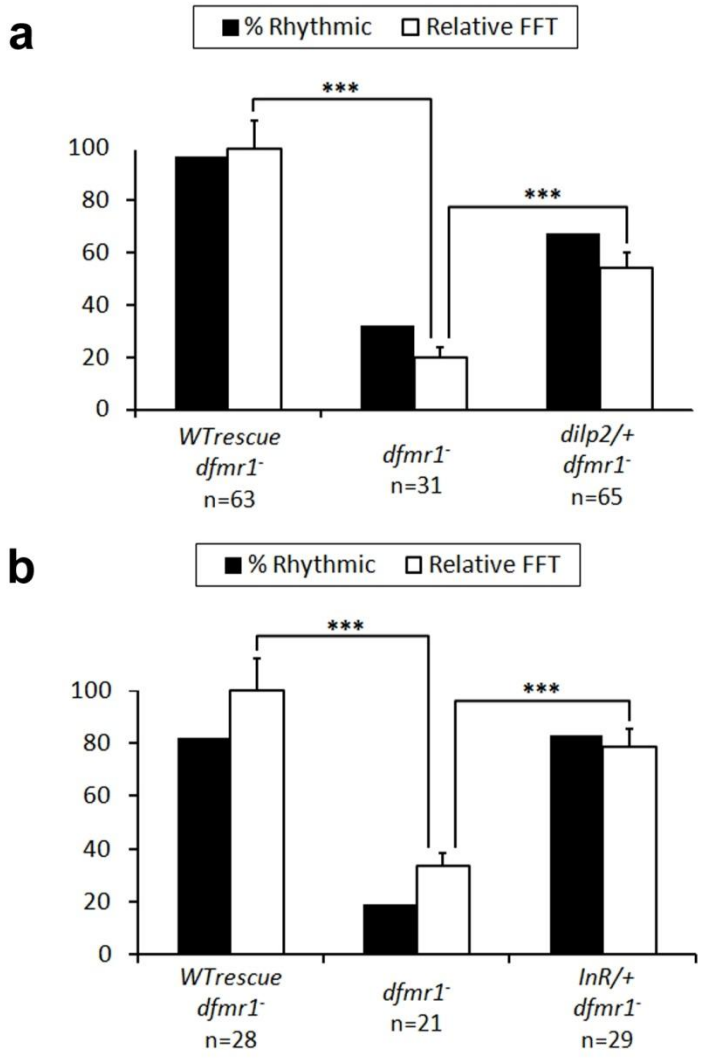

Figure 3-1. Ubiquitous decrease of insulin signaling rescues memory in *dfmr1* mutants. Panels show the percentage of rhythmic flies (black) and relative FFT values (white) for genetic combinations testing the effect of reducing insulin signaling in *dfmr1* mutants on circadian behavior. **(a)** Circadian behavior (as indicated by increased percentage of rhythmic flies and increased relative FFT) of *dfmr1* mutants with the *WTrescue* transgene or with one copy of a null allele of *dilp2* (*dilp2*/+; *dfmr1*$^{-}$) is significantly improved relative to *dfmr1* mutant controls, p<0.001. **(b)** Circadian behavior of *dfmr1* mutants with the *WTrescue* transgene or with one copy of a mutant allele of the insulin receptor (*InR*/+; *dfmr1*$^{-}$) is significantly improved relative to *dfmr1* mutant controls, p<0.001. Rhythmicity was determined by FFT (.01 or higher) and visual inspection of the actogram and periodogram. Statistical significance is denoted with asterisks (*p<0.05, ** p<0.01, ***p<0.001). Statistical significance was determined by a Kruskal-Wallis test and Dunn's post-test. Graphs depict mean ± s.e.m.

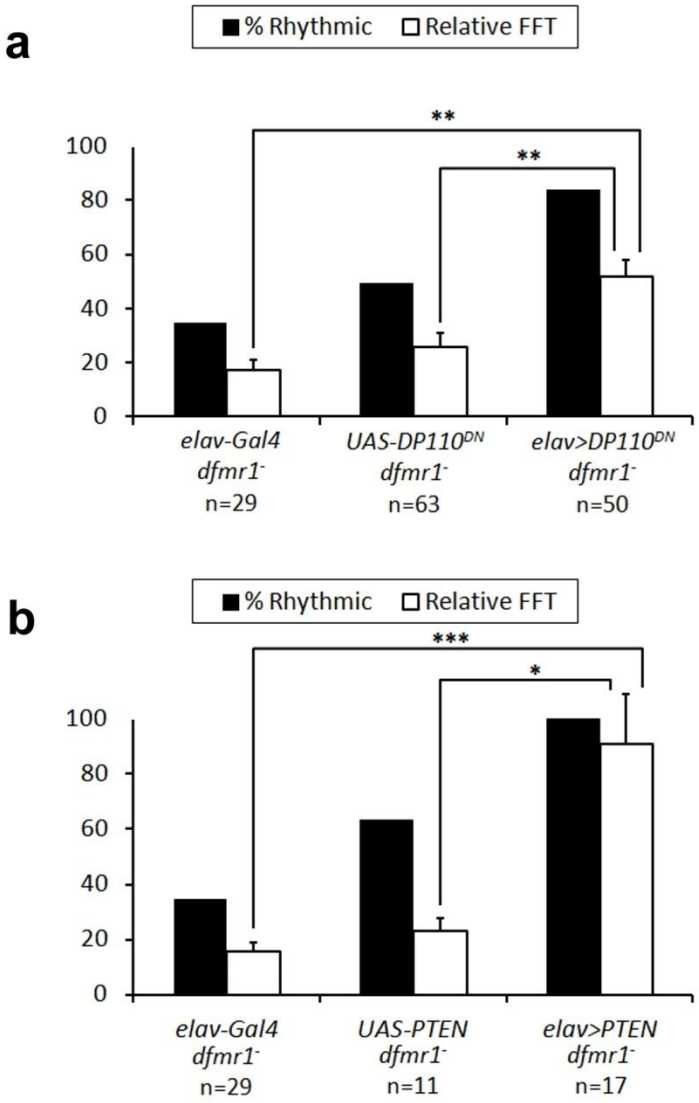

Figure 3-2. Pan-neuronal reduction of insulin signaling in *dfmr1* mutants rescues circadian rhythmicity. Panels show the percentage of rhythmic flies (black) and relative FFT values (white) for genetic combinations testing the effect of reducing insulin signaling in *dfmr1* mutants on circadian behavior. **(a)** Circadian behavior of *dfmr1* mutants with both *elav-Gal4* and *UAS-DP110^{DN}* (*elav>DP110^{DN}; dfmr1^{-}*) is significantly improved relative to *dfmr1* mutants with either transgene alone (*elav-Gal4; dfmr1^{-}*) and (*UAS-DP110^{DN}; dfmr1^{-}*), p<0.01. **(b)** Circadian behavior of *dfmr1* mutants with both *elav-Gal4* and *UAS-PTEN* (*elav>PTEN; dfmr1^{-}*) is significantly improved relative to *dfmr1* mutants with either transgene alone (*elav-Gal4; dfmr1^{-}*) and (*UAS-PTEN; dfmr1*), p<0.001 and p<0.05 respectively. Rhythmicity was determined by FFT (.01 or higher) and visual inspection of the actogram and periodogram. Statistical significance is denoted with asterisks (*p<0.05, ** p<0.01, ***p<0.001). Statistical significance was determined by a Kruskal-Wallis test and Dunn's post-test. Graphs depict mean ± s.e.m.

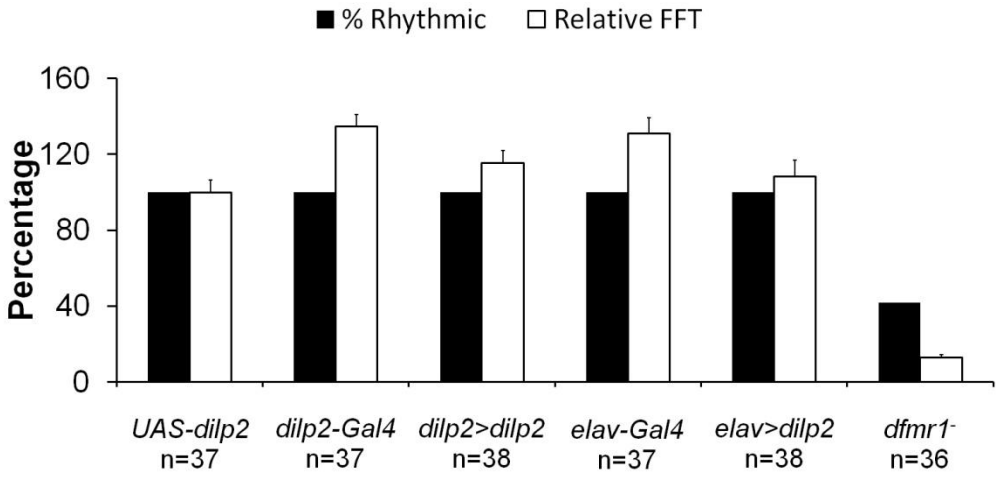

Figure 3-3. Overexpression of Dilp2 does not cause arrhythmicity in wild-type flies. Panels show the percentage of rhythmic flies (black) and relative FFT values (white) for genetic combinations testing the effect on of increasing insulin signaling on circadian behavior in otherwise wild-type flies. Overexpression of Dilp2 in either the IPCs or in all neurons does not result in decreased circadian rhythmicity. Rhythmicity was determined by FFT (.01 or higher) and visual inspection of the actogram and periodogram. Statistical significance is denoted with asterisks (*p<0.05, ** p<0.01, ***p<0.001). Statistical significance was determined by a Kruskal-Wallis test and Dunn's post-test. Graphs depict mean ± s.e.m.

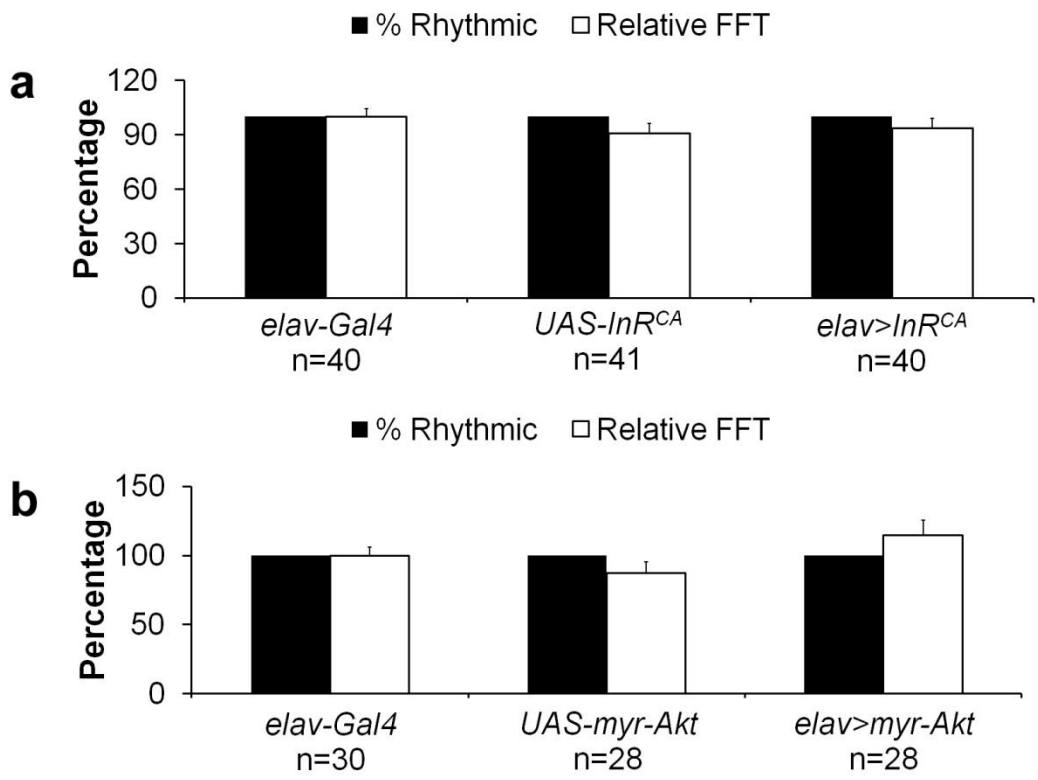

Figure 3-4. Pan-neuronal insulin signaling increase does not affect circadian behavior in wild-type flies. Panels show the percentage of rhythmic flies (black) and relative FFT values (white) for genetic combinations testing the effect of increasing insulin signaling on circadian behavior in wild-type flies. **(a)** Pan-neuronal expression of a constitutively active insulin receptor in a wild-type background does not reduce circadian rhythmicity. **(b)** Pan-neuronal expression of a constitutively active Akt in wild-type flies does not negatively affect circadian rhythmicity. Rhythmicity was determined by FFT (.01 or higher) and visual inspection of the actogram and periodogram. Statistical significance is denoted with asterisks (*p<0.05, ** p<0.01, ***p<0.001). Statistical significance was determined by a Kruskal-Wallis test and Dunn's post-test. Graphs depict mean ± s.e.m.

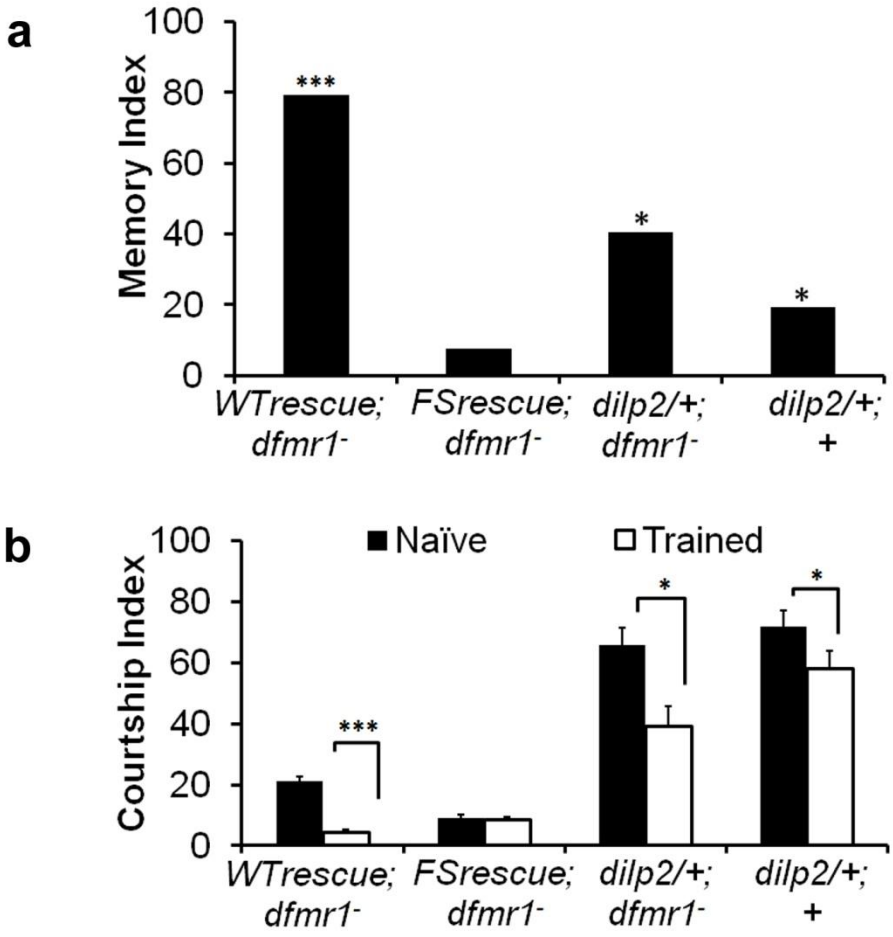

Figure 3-5. Reducing *dilp2* levels rescues courtship-based STM defects in *dfmr1* mutants. STM in the courtship paradigm is presented as **(a)** memory index (MI), which conveys the difference between the courtship indices (CIs) of trained and untrained flies in a single value (MI = $(CI_{naive}-CI_{trained})/CI_{naive}$), and **(b)** as separate CIs. If memory is present, the CI of trained flies will be reduced compared to the CI of naïve flies, indicating that the trained fly remembers thenegative experience of rejection by an unreceptive female. STM is rescued by reduction of *dilp2* in *dfmr1* mutants, $p<0.05$. *FSrescue* represents a frame-shifted version of the *dfmr1* open reading frame. N=25-31. Data were subjected to an arcsine transformation to obtain a normal distribution, and then statistical significance was determined by ANOVA with pre-planned comparisons. Statistical significance is denoted with asterisks (*$p<0.05$, ** $p<0.01$, ***$p<0.001$). Graphs depict mean ± s.e.m. These data were collected by Brian Schoenfeld and Sean McBride.

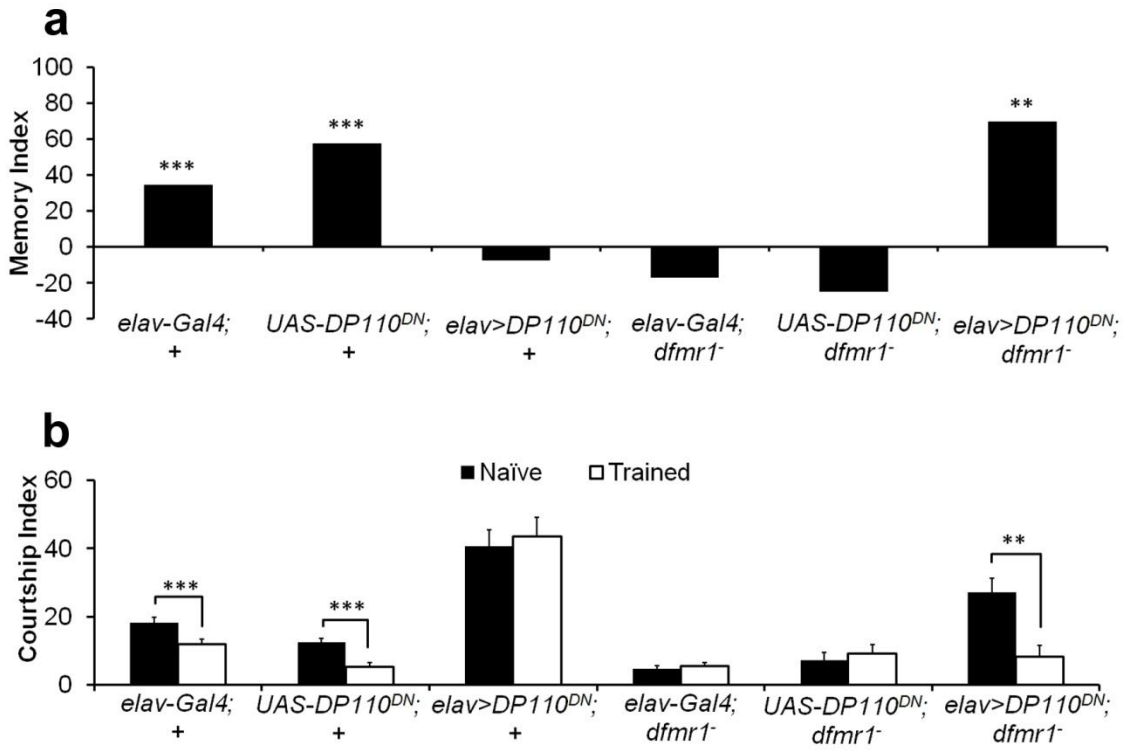

Figure 3-6. Pan-neuronal reduction of PI3K activity rescues STM defects in *dfmr1* mutants. STM in the courtship paradigm is presented as **(a)** memory index (MI), which conveys the difference between the courtship indices (CIs) of trained and untrained flies in a single value (MI = $(CI_{naive}-CI_{trained})/CI_{naive}$), and **(b)** as separate CIs. If memory is present, the CI of trained flies will be reduced compared to the CI of naïve flies, indicating that the trained fly remembers the negative experience of rejection by an unreceptive female. STM is rescued by pan-neural expression of $DP110^{DN}$, $p<0.01$. N=22-91. Data were subject to an arcsine transformation to obtain a normal distribution, and then statistical significance was determined by ANOVA with pre-planned comparisons. Statistical significance is denoted with asterisks (*$p<0.05$, ** $p<0.01$, ***$p<0.001$). Graphs depict mean ± s.e.m. These data were collected by Brian Schoenfeld and Sean McBride.

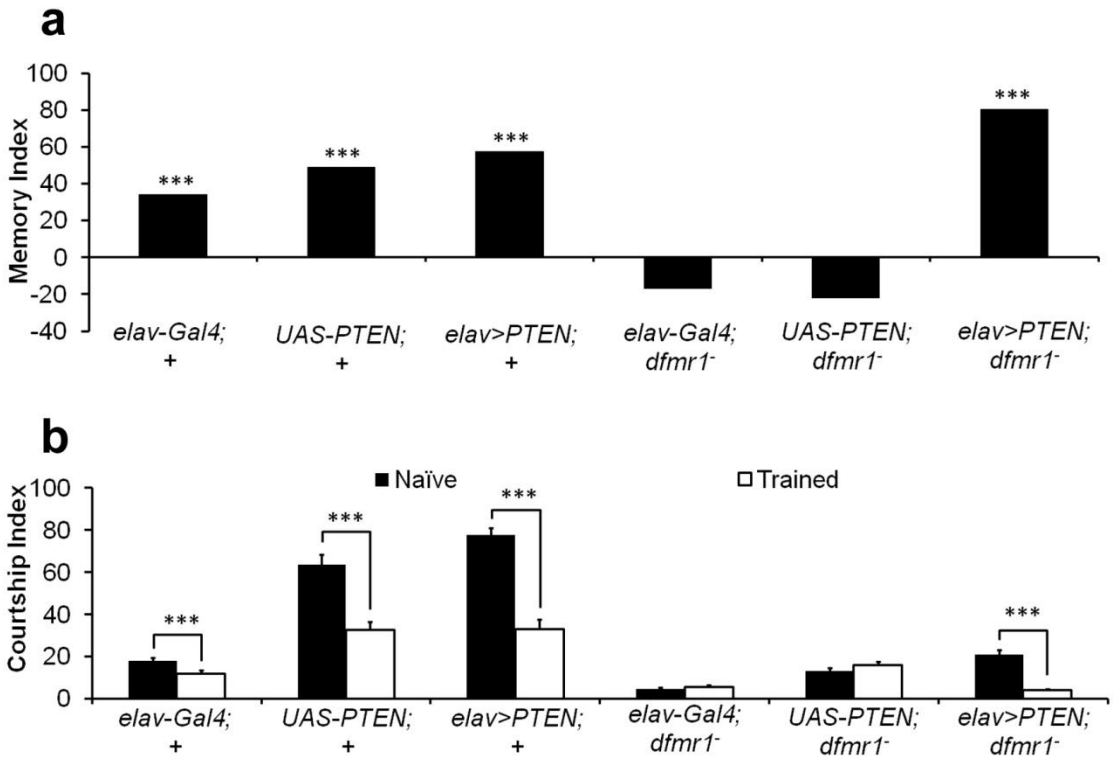

Figure 3-7. Pan-neuronal over-expression of PTEN rescues STM defects in *dfmr1* mutants. STM in the courtship paradigm is presented as **(a)** memory index (MI), which conveys the difference between the courtship indices (CIs) of trained and untrained flies in a single value (MI = $(CI_{naive}-CI_{trained})/CI_{naive}$), and **(b)** as separate CIs. If memory is present, the CI of trained flies will be reduced compared to the CI of naïve flies, indicating that the trained fly remembers the negative experience of rejection by an unreceptive female. STM is rescued by pan-neural expression of PTEN, $p<0.001$. N=28-91. Data were subject to an arcsine transformation to obtain a normal distribution, and then statistical significance was determined by ANOVA with pre-planned comparisons. Statistical significance is denoted with asterisks (*$p<0.05$, ** $p<0.01$, ***$p<0.001$). Graphs depict ± s.e.m. These data were collected by Brian Schoenfeld.

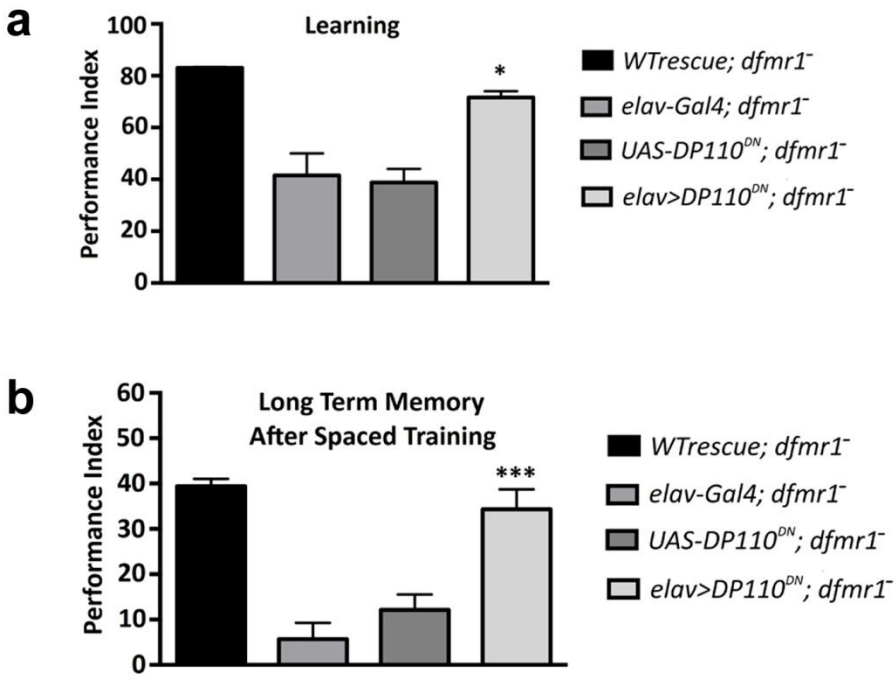

Figure 3-8. Reducing PI3K activity in *dfmr1* mutants rescue learning and LTM in the olfactory conditioning paradigm. Performance index (PI) represents the percent of flies which avoid the shock-conditioned odor. (PI = % of flies avoiding shock-conditioned odor - % of flies moving toward shock conditioned odor). **(a)** *Dfmr1* mutants expressing $DP110^{DN}$ pan-neuronally show rescue of learning (N=4, p=0.0235) and **(b)** memory, (N=8, p=0.0005). Statistical significance was determined by an ANOVA with Bonferroni correction and a Tukey post-test. Statistical significance is denoted with asterisks (*p<0.05, ** p<0.01, ***p<0.001). Graphs depict mean ± s.e.m. These data were collected by Daniel Chambers and Francois Bolduc.

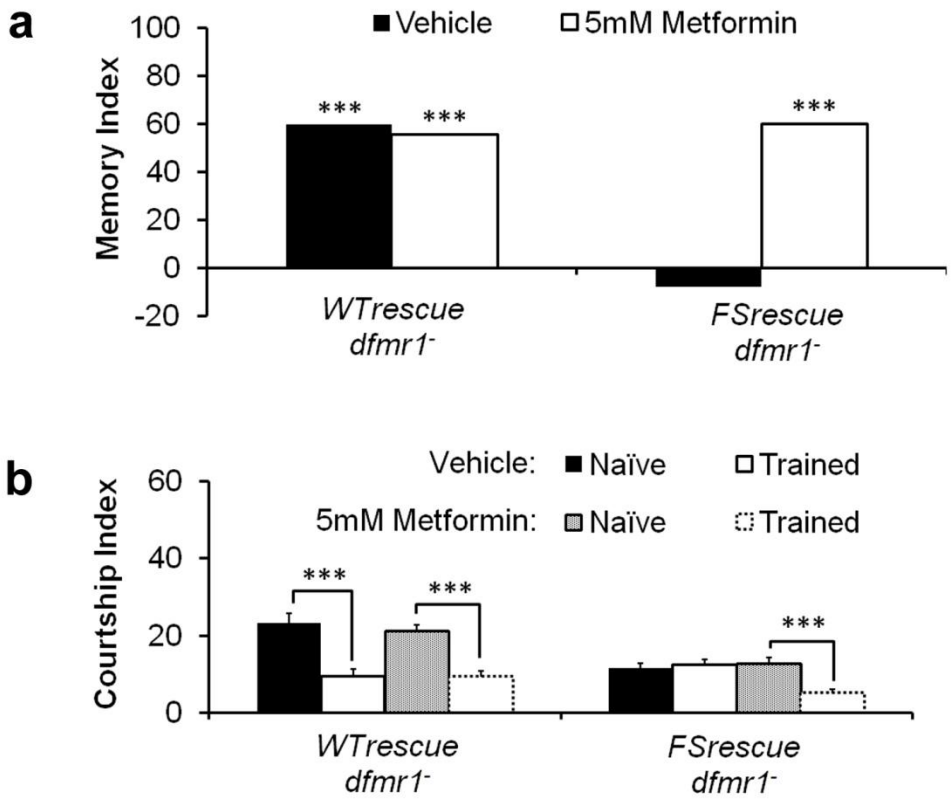

Figure 3-9. Adulthood metformin treatment rescues the STM defect in *dfmr1* mutant flies. STM in the courtship paradigm is presented as **(a)** memory index (MI), which conveys the difference between the courtship indices (CIs) of trained and untrained flies in a single value (MI = $(CI_{naive}-CI_{trained})/CI_{naive}$), and **(b)** as separate CIs. If memory is present, the CI of trained flies will be reduced compared to the CI of naïve flies, indicating that the trained fly remembers the negative experience of rejection by an unreceptive female. Metformin treatment restores STM to *dfmr1* mutants, p<.001 N=36-86. Data were subject to an arcsine transformation to obtain a normal distribution, and then statistical significance was determined by ANOVA with pre-planned comparisons. Statistical significance is denoted with asterisks (*p<0.05, ** p<0.01, ***p<0.001). Graphs depict mean ± s.e.m.

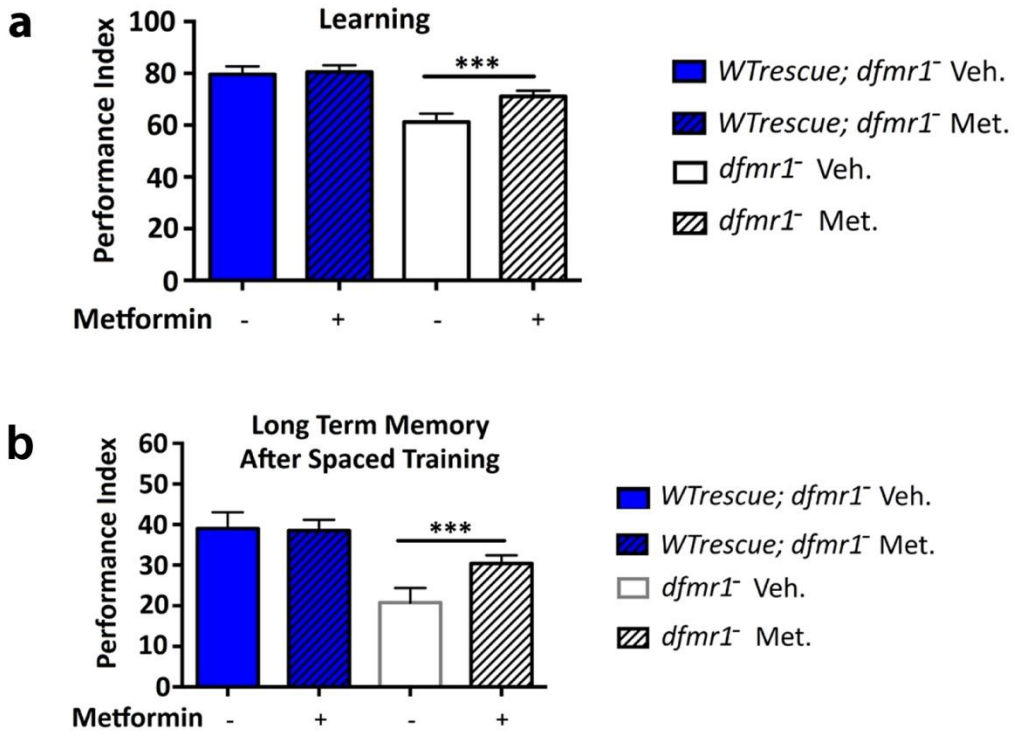

Figure 3-10. Acute metformin treatment rescues learning and LTM defects of *dfmr1* mutants in the olfactory conditioning paradigm. Performance index (PI) represents the percent of flies which avoid the shock-conditioned odor. (PI = % of flies avoiding shock-conditioned odor - % of flies moving toward shock conditioned odor). Metformin improves **(a)** learning ($p<0.0001$). (N= 6) and **(b)** long-term memory in *dfmr1* mutants. (N=8, $p <0.00018$). Statistical significance was determined by an ANOVA with Bonferroni correction and a Tukey post-test. Statistical significance is denoted with asterisks (*$p<0.05$, ** $p<0.01$, ***$p<0.001$). Graphs depict mean ± s.e.m. These data were collected by Daniel Chambers and Francois Bolduc.

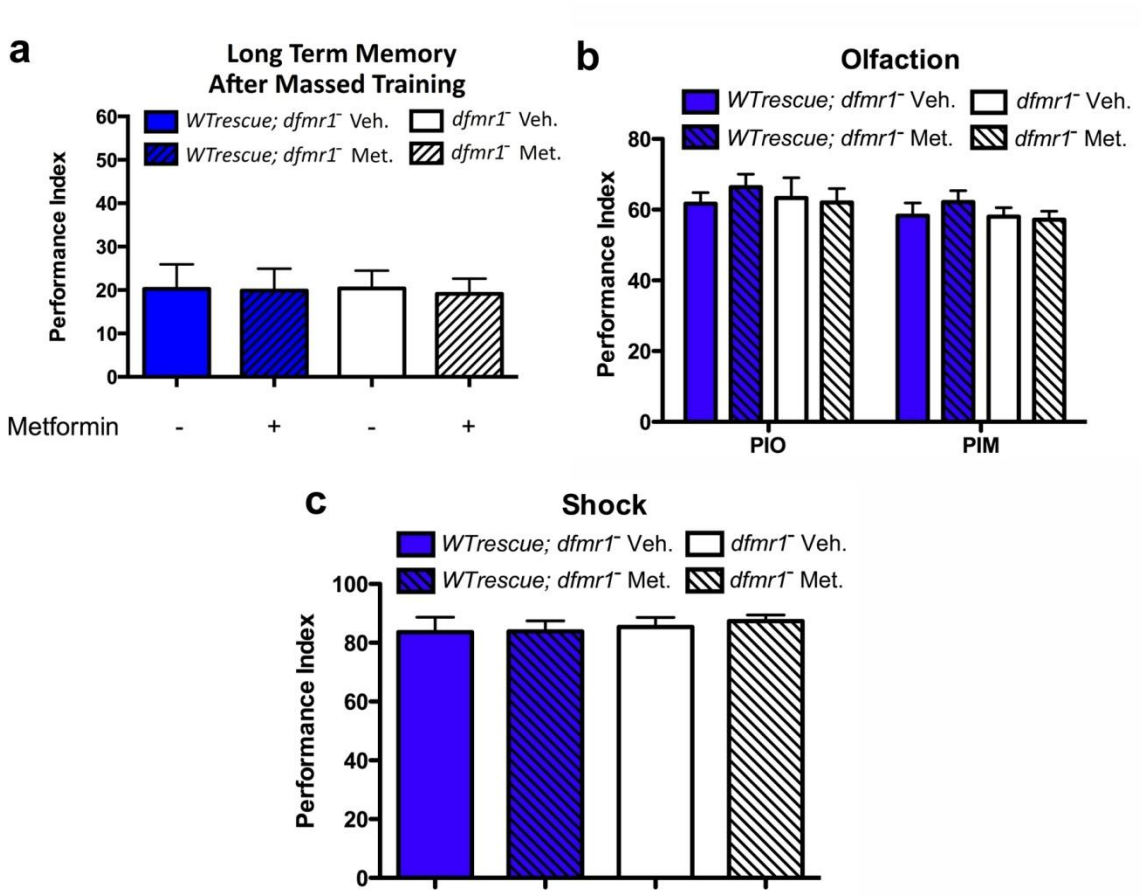

Figure 3-11. *Dfmr1* mutants do not display defects in LTM after massed training, olfaction or shock sensitivity. Performance index (PI) represents the percent of flies which avoid the shock-conditioned odor. (PI = % of flies avoiding shock-conditioned odor - % of flies moving toward shock conditioned odor). **(a)** One-day memory after massed training does not differ between groups. (n = 8 PIs per group). **(b)** *Dfmr1* mutants as well as genetic controls containing the *WTrescue* genomic rescue fragment show normal shock avoidance with vehicle and 1mM metformin treatment. (ANOVA p=0.3043; n= 6 PI per genotype). **(c)** *Dfmr1* mutant *Drosophila* as well as genetic controls containing the *WTrescue* genomic rescue fragment present with normal avoidance to the odors (avoidance to OCT versus air=PIO or avoidance to MCH versus air=PIM) used in the classical conditioning experiments. (ANOVA p=0.9936; n= 6 PI per genotype). Statistical significance was determined by an ANOVA with Bonferroni correction and a Tukey post-test. Statistical significance is denoted with asterisks (*p<0.05, ** p<0.01, ***p<0.001). All error bars depict s.e.m. These data were collected by Daniel Chambers and Francois Bolduc.

Materials and Methods:

Fly stocks and maintenance

Fly stocks were maintained on standard cornmeal-molasses medium. Fly strains containing the *dilp2* and InR^{5545} mutations were obtained from Bloomington Stock Center (stock numbers 30881 and 11661) (FERNANDEZ *et al.* 1995; GRONKE *et al.* 2010). Fly stocks carrying the *UAS-*$DP110^{DN}$ and *UAS-PTEN* transgenes were obtained from M. Birnbaum (LEEVERS *et al.* 1996; HUANG *et al.* 1999). The *elav-Gal4* transgene was derived from Bloomington Stock number 8765. The $dfmr1^{3}$allele and *WTrescue* are previously described in (DOCKENDORFF *et al.* 2002). The *dilp2-Gal4* driver was obtained from Eric Rulifson (RULIFSON *et al.* 2002). *UAS-myr-Akt* and *UAS-*InR^{CA} fly stocks were obtained from the Birbaum lab. Stocks containing the *UAS-dilp2* transgene were obtained from the Sehgal lab. Flies used for the olfactory learning assay were outcrossed to the *w1118(isoCJ1)* flies.

Circadian Behavior Assay

Flies intended for circadian rhythmicity analysis were raised at 25°C on a L:D cycle. Male flies were collected at 0-3 days of age and maintained on standard fly food in an L:D incubator for entrainment. After 3-5 days, individual flies were loaded into 2% agar, 5% sucrose tubes that were subsequently placed in activity monitors (TriKinetics) and maintained in dark:dark conditions for 10 days.

Data were collected in 5 minute bins and analyzed with Clock Lab software (Actimetrics) to obtain period and rhythmicity values. Rhythmicity was determined by fast fourier transform (FFT) analysis (with rhythmicity defined as a FFT value of 0.01 or more) as well as visual inspection of the actogram and periodogram. Significant differences in average FFT values between genotypes were determined using a Kruskal-Wallis test followed by a Dunn's post-test (GraphPad, InStat). Relative FFT was calculating by dividing the average FFT value of the

depicted genotype by the average FFT value of the wild-type control: Relative FFT = $FFT_{depicted}/FFT_{wild\text{-}type}*100$.

Short-term memory assay

Virgin male flies were collected under CO_2 anesthesia every four hours and maintained on standard fly food in small all-male groups at 25°C in L:D until testing. Virgin X^X, *yf* test females were collected on the day of eclosion and kept in food vials. Flies were aged in a 12hr:12hr L:D incubator before behavioral training and testing was performed during the relative light phase. All male subjects were transferred to fresh control food the day before testing and assigned to random groups for behavioral training and testing. All training and testing was performed blind to genotype and treatment. A courtship index (CI) was calculated following testing as the percentage of total observation time spent courting. CIs of tested males were subjected to arcsin square root transformations to approximate normal distributions. ANOVAs were performed on pre-planned pair-wise comparisons of arcsin transformed data to get critical p-values.

For data not normal after transformation, the Mann-Whitney test was used to generate p-values (SIEGEL 1957). Statistics were performed using Statview 3.0 and Prism. Memory Index (MI) = $(CI_{naive}\text{-}CI_{trained})/CI_{naive}$ (KELEMAN *et al.* 2007).

For drug treatment, flies were raised standard fly food until eclosion. Adult male flies were collected within 4 hours of eclosion and placed on food containing 5mM metformin or vehicle (water). One day before STM testing flies were moved to individual vials containing standard fly food.

Pavlovian olfactory learning and memory

Flies were raised at 22°C and placed at 25°C overnight prior to behavioral experiments. Adult *Drosophila* 1-3 days old were trained and tested with the classical conditioning procedure.

About 100 flies were trapped inside a training chamber covered with an electrifiable copper grid. Flies were allowed 90 seconds to acclimate and then were exposed sequentially to two odors, 3-octanol (OCT) and 4- methylcyclohexanol (MCH), carried through the chamber in a current of air. Relative concentrations of OCT and MCH were adjusted so that naïve flies distributed themselves 50:50 in the T-maze. Flies first were exposed for 60 seconds to the conditioned stimulus (CS+; either OCT or MCH, depending on the odor the flies were shocked to in the first step), during which time they received the unconditioned stimulus (US; twelve 1.25 seconds pulses of 60V DC electric shock at 5 second interpulse intervals). After the CS+ presentation, the chamber was flushed with fresh air for 45 seconds. Then flies were exposed for 60 seconds to a second, control stimulus (CS-; either MCH or OCT), which was not paired with electric shock. After the CS- presentation, the chamber was again flushed with fresh air for 45 seconds.

To test for conditioned odor avoidance after classical conditioning, flies were moved to the choice point of the T-maze. Ninety seconds later, the flies were exposed to two converging current of air one carrying OCT, the other MCH, from opposite arms of the T-maze. Flies were allowed to choose between the CS+ and CS- for 120 seconds, at which time they were trapped inside their respective arms of the T-maze, anesthetized and counted.

For long-term memory testing, 1-3 days old adult flies were subjected to classical (Pavlovian) olfactory conditioning for 10 training sessions without a rest interval (massed training) or to 10 training sessions with 15 minutes rest between each training session (spaced training). After training, flies were stored at 18^{o}C and then conditioned responses were tested after a 24-hour retention interval at 25°C.

For both learning and long-term memory, two groups of flies were trained and tested in one complete experiment. The CS+ was OCT and the CS- was MCH for one group; the CS+ was MCH and the CS- was OCT for the second group. The performance index (PI) was calculated as the average of the fraction of the population avoiding the shock-associated odor minus the fraction avoiding the control odor for each group of flies trained in one experiment. In other words,

the PI enumerates the distribution of flies in the T-maze as a normalized “percent correctly avoiding the shock-paired odor” and ranges from 0 for a 50:50 distribution to 100 for a 100:0 distribution.

Data from an experiment were subjected to a one-way ANOVA (JMP from SAS, Inc.), followed by planned pair-wise comparisons. An alpha = 0.05 was corrected for multiple comparisons using Bonferroni. Post-test analysis was performed with the Tukey test.

For drug treatment, flies were placed in vials overnight at 25°C and 70% humidity with Whatman filter paper containing 200 uL of either 1mM metformin or vehicle (water) and 5% sucrose.

Olfactory acuity and shock reactivity were assessed. Odor avoidance at the concentrations used for the conditioning experiments was quantified in mutant and control flies. Naïve flies were placed in the T-maze and given a choice between an odor (OCT or MCH) and air. The odor is naturally aversive, and flies usually avoided the T-maze arm containing the odor (OCT or MCH) by running into the opposite arm (air). After the flies distributed themselves for 2 minutes, they were trapped, anesthetized and counted. For shock reactivity, flies were given a choice between an electrified grid in one T-maze arm and an unconnected grid in the other. After the flies distributed themselves for 2 minutes, they were trapped, anesthetized and counted.

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Chapter 4: Examination of the developmental role of the insulin signaling pathway in *dfmr1* mutant phenotypes

*Portions of this chapter are excerpted with modification from Monyak RE, Emerson D, Schoenfeld BP, Zheng X, Chambers DB, Rosenfelt C, Langer S, Hinchey P, Choi CH, McDonald TV, Bolduc FV, Sehgal A, McBride SM, Jongens TA. 2016. Insulin signaling misregulation underlies circadian and cognitive deficits in a *Drosophila* fragile X model. *Mol Psychiatry.* doi: 10.1038/mp2016.51 [Epub ahead of print]

Abstract:

Fragile X syndrome (FXS) is a developmental disease that occurs due to loss of expression of *FMR1*. In the previous chapters, we showed that increased insulin signaling in *dfmr1* mutants contributes to both circadian and memory deficits. Interestingly, we also found that acute administration of the insulin signaling normalizing drug metformin was able to rescue both courtship-based and olfactory based memory in *dfmr1* mutants. We therefore wanted to understand the importance of insulin signaling during development in *dfmr1* mutants.

We used the temperature-sensitive Gal80 ($Gal80^{ts}$) to restrict insulin signaling reduction to different stages of development, and examined circadian behavior and memory. We found that insulin signaling reduction was required during the pupal stage to promote circadian rhythmicity, but was not required until adulthood to promote memory. We also tested the developmental requirements of insulin signaling normalization by treating with metformin at different developmental stages. We failed to rescue circadian behavior with metformin treatment at any time point, but were able to rescue courtship-based memory with treatment in both development alone and adulthood alone. Olfactory-based memory was less amenable to treatment, and was only rescued by acute adulthood metformin treatment. These findings highlight the divergent pathways through which insulin signaling regulates different behaviors in *Drosophila.*

Introduction:

Fragile X syndrome (FXS) is a diseases that begins in childhood and continues for the entire lifespan of the affected patient (TURK 2011). Indeed, FXS usually begins early in embryogenesis when a *Fmr1* gene with an expanded 3' untranslated region is methylated and ceases to be expressed (OOSTRA AND WILLEMSEN 2009). For the 1% of patients who have other mutations or deletions of *Fmr1,* the disease begins even earlier (GARBER *et al.* 2008). FXS is a developmental disease, and evidence suggests *FMR1* has a critical role in neurogenesis and brain development (CASTREN *et al.* 2005; CALLAN *et al.* 2010; GUO *et al.* 2011; TELIAS *et al.* 2013). It is likely that many of the symptoms seen in FXS patients have their origins early in development, probably before the patients were even born.

Individuals with FXS suffer from low IQ, attention deficit hyperactivity disorder, epilepsy, autism, and frequently disordered sleep (GARBER *et al.* 2008; KIDD *et al.* 2014). These difficulties generally present themselves in early childhood, though some do not appear until adolescence or later (TURK 2011). However, it is not known if the neural or pathological underpinnings of these symptoms originate immediately before the appearance of the symptoms, or if their origins are much earlier. To address these questions, we turned to our *Drosophila* FXS model. Since *dfmr1* mutant flies exhibit memory and circadian defects, we can attempt to understand the developmental origins of these defects and the signaling pathways and circuitry involved.

In the previous chapters, we showed that *dfmr1* expression in the insulin-producing cells (IPCs) is important for both circadian rhythmicity and memory in both the conditioned courtship paradigm and the olfactory conditioning paradigm. We also showed that insulin signaling is increased in the brains of *dfmr1* mutant flies, and that we can rescue the circadian and memory defects of these flies by genetically reducing insulin signaling either ubiquitously or solely in neurons. We determined that treating flies with the insulin signaling normalizing drug, metformin, was able to acutely rescue memory in adulthood. This latter result indicates that some of these phenotypes can be rescued in adulthood, however, a full examination of the developmental origins of circadian arrhythmicity and memory defects in *dfmr1* mutants has not been conducted.

The answers to these questions will help us untangle the neural circuits and signaling pathways essential for normal behavior in *dfmr1* mutants.

Fortunately, several genetic tools are available that allowed us to address the question of when insulin signaling must be normalized for the development of typical behavior. A temperature-sensitive Gal80 ($Gal80^{ts}$) can be used in conjunction with the Gal4/UAS system to modulate expression of a gene-of-interest. At permissive temperatures, usually around 18°C, the $Gal80^{ts}$ will repress Gal4 so the UAS is not expressed. At restrictive temperatures, usually around 29°C, the $Gal80^{ts}$ will cease to function, relieving the repression of Gal4 so that it activates the UAS, resulting in expression of the gene-of-interest (McGuire *et al.* 2004). This system can be used to test the temporal requirements of *UAS-DP110*^{DN} and *UAS-PTEN* transgene expression for rescue of circadian behavior and memory.

The question of the developmental origins of circadian and memory defects in *dfmr1* mutants can further be addressed by conducting a more extensive study of metformin administration. In Chapter 3, we showed that metformin treatment solely in adulthood could rescue both courtship-based and olfactory-based memory, but we did not examine the effects of developmental treatment. Knowledge of the temporal requirements of metformin treatment give us another picture of the mechanisms underlying circadian behavior and courtship, and may hint at the mechanisms by which metformin acts to rescue behavior.

In this chapter, we examine the temporal requirements of insulin signaling reduction and metformin administration for rescue of circadian behavior and memory. We found that normalization of insulin signaling had to occur during the pupal period in *dfmr1* mutants to restore circadian rhythmicity. Normalization of insulin signaling solely during adulthood was not sufficient to rescue circadian behavior. Conversely, insulin signaling normalization during adulthood was sufficient to restore both STM in the conditioned courtship paradigm, and learning in the olfactory conditioning paradigm. These results suggest that insulin signaling influences circadian behavior and memory through fundamentally different pathways.

Similarly, we found that circadian behavior and memory were affected differently by metformin treatment. While adulthood metformin treatment rescued both forms of memory in *dfmr1* mutant flies, no combination of developmental and adulthood treatment with metformin rescued circadian behavior. Interestingly, we also found differences in the developmental specifications for metformin treatment between courtship-based memory and olfactory-based memory, as developmental metformin treatment rescued courtship-based memory but did not rescue olfactory-based memory. These results suggest that insulin signaling affects the two forms of memory through different pathways.

Altogether, our results indicate that the temporal requirements for insulin signaling regulation are different for circadian behavior and memory, suggesting that abnormal insulin signaling disrupts these two types of behaviors by different mechanisms. We also show that metformin treatment is only effective to rescue memory, and that it has different temporal requirements for olfactory conditioned memory and courtship conditioned memory, again suggesting that the mechanisms by which metformin rescues these two types of memory are different.

Results:

Reduction of insulin signaling during the pupal period is required for normal circadian rhythmicity in dfmr1 mutants

To more directly explore the temporal requirements of insulin signaling normalization for circadian rhythmicity, we used the $Gal80^{ts}$ repressor of Gal4 to initiate insulin signaling reduction at different developmental stages. We used this system to test the effect of initiating insulin signaling reduction at the beginning of the pupal stage, or within 24 hours of eclosion. Interestingly, we found that when insulin signaling was reduced using $DP110^{DN}$, the reduction had to occur during the pupal period to achieve rescue of circadian behavior (Figure 4-1a & b). A similar result was observed when insulin signaling was reduced by PTEN overexpression, though comparison to one control did not reach significance (Figure 4-2a & b). These results suggest that

normalization of insulin signaling during the pupal period is necessary for rescue of circadian behavior.

Since metformin treatment in adulthood alone rescued both olfactory-based and courtship-based memory, we used the Gal80^{ts} to test whether initiating reduction of insulin signaling in adulthood would also be sufficient to rescue memory in these assays. Adulthood-restricted reduction of insulin signaling using either pan-neuronal DP110^{DN} or PTEN expression rescued STM in the conditioned courtship paradigm (Figure 4-3a & b). Similarly, adulthood reduction of insulin signaling by pan-neuronal DP110^{DN} expression was sufficient to rescue learning in the olfactory-based memory paradigm (Figure 4-4). Together, these results indicate that while physiological insulin signaling reduction is sufficient to rescue memory in the olfactory-based and courtship-based paradigms, reduction of insulin signaling during the pupal period is essential to rescue circadian behavior in *dfmr1* mutant flies. These findings suggest that the circadian and memory deficits seen in *dfmr1* mutant flies are ultimately caused by separate defects that are both affected by insulin signaling misregulation.

Metformin treatment over several temporal stages rescues memory

Following the result that acute metformin treatment rescued defects in both courtship-based and olfactory-based memory, we examined how metformin treatment during development and/or adulthood affected both memory and circadian behavior. While we were unable to rescued circadian rhythmicity with any combination of developmental and adulthood metformin treatment (Figure 4-5, Figure 4-6), we found that STM was amenable to rescue with several combinations of temporally-restricted metformin treatment (Figure 4-7, Figure 4-8). Intriguingly, we were able to rescue STM in the conditioned courtship paradigm with developmental treatment alone (Figure 4-7, Figure 4-8). In contrast, developmental metformin treatment was unable to rescue learning or LTM in the olfactory conditioning paradigm (data not shown). These results suggest that although courtship-based and olfactory-based memory are both forms of memory, their developmental requirements for normalized insulin signaling are different.

Discussion:

Here we show that different development pressures govern memory and circadian behavior in *dfmr1* mutants, even though they are regulated by a common pathway. Specifically, we show that reduction of insulin signaling must occur during the pupal stage to rescue circadian rhythmicity, but is not required until adulthood to rescue memory. We also found that metformin treatment could not rescue circadian rhythmicity at any time during development. In contrast, metformin treatment was able to rescue STM when administered at all combination of developmental time points. Finally, we found that administration of metformin during adulthood, but not development, could rescue learning and LTM in the olfactory conditioning paradigm.

The result that circadian behavior could be rescued by reducing insulin signaling beginning in the pupal period, but not beginning in adulthood, indicates that regulated insulin signaling is required during the pupal period for proper circadian behavior. This finding suggests that some critical event occurs during the pupal period for which well-regulated insulin signaling is essential. To determine what this event could be, we would need to closely focus on the pupal period and examine changes that take place in neural wiring during that time. Unfortunately, not enough is known about the precise role of insulin signaling in development during this period to make any specific speculations about what could be happening. However, we should examine insulin signaling during the pupal stage and determine if insulin signaling is increased in the brains of *dfmr1* mutants. This experiment will allow us to establish how insulin signaling changes during the pupal period in *dfmr1* mutants and allow us to identify regions of the brain to observe more closely.

We also found that reduction of insulin signaling is not required for memory until adulthood. Specifically, reduction of insulin signaling in adulthood rescued STM in the conditioned courtship paradigm, and rescued learning in the olfactory conditioning paradigm. Our ability to rescue memory with adulthood treatment alone is not entirely surprising, since it has previously been reported that memory defects in *dfm1* mutant flies can acutely be rescued by drug treatment (McBride *et al.* 2005; Bolduc *et al.* 2010). Of course, it is still interesting that insulin signaling

specifically can rescue memory in adulthood, because it indicates that it is physiological misregulation of insulin signaling, and not developmental misregulation, that causes memory defects. The deficit is thus more likely to be directly caused by the current insulin signaling environment, rather than caused indirectly by an event occurring during development that is disrupted by abnormal insulin signaling. The finding that we are unable to rescue circadian rhythmicity by adulthood treatment suggests the converse: that circadian arrhythmicity is likely due to an event occurring during development that is disrupted in *dfmr1* mutants due to aberrant insulin signaling.

We identified the time periods during which we had to initiate reduction of insulin signaling to rescue circadian behavior and memory. Ideally, we would like to determine both the start and end points for the window during which insulin signaling normalization is required for normal circadian behavior and memory. Unfortunately, we were unable to identify time point at which insulin signaling reduction ceases to be necessary. Although we could shift the flies back to the permissive temperature for $Gal80^{ts}$, thus abolishing further expression of the *UAS-DP110*^{DN} or *UAS-PTEN* transgenes, we have no way of measuring how long the already-expressed proteins continue to function before its levels are reduced due to degradation. However, if antibodies become available that would allow us to visualize $DP110^{DN}$ or PTEN levels in *Drosophila,* we could use them to address some interesting questions left unanswered by this experiment. First, it would be informative to learn whether insulin signaling normalization in only the pupal stage (and not adulthood as well) is sufficient to restore circadian rhythmicity. If we determined that pupal insulin signaling reduction was sufficient to improve rhythmicity, it would be of great value to determine precisely when during the pupal period normalization of insulin signaling is most important. This information might hint at the identity of mechanism through which increased insulin signaling disrupts circadian behavior, and would at least give us a narrower time period on which to focus. It would also be interesting to address when insulin signaling normalization is required for memory. We showed that metformin treatment in development alone rescued STM in the conditioned courtship paradigm. This result suggests that insulin signaling normalization in

development alone might also be able to rescue memory. This experiment would help us determine if how metformin rescues memory, because if genetic insulin signaling normalization and pharmacological insulin signaling normalization were required at the same time, it would suggest that metformin might act more directly on insulin signaling.

Our developmental timing experiment had flaws aside from our inability to identify an endpoint for the insulin signaling normalization requirement. It was complicated by effects on circadian behavior caused by culture at unfavorable temperatures. Raising flies constantly at 18°C or 29°C seemed to have detrimental effects on circadian behavior, and keeping flies at these temperatures for any length of time had a detrimental effect on courtship behavior. We therefore used 22°C as the restrictive temperature and 27°C as the permissive temperature for our courtship and olfactory experiments. These complications exhibit the downside of the Gal80^{ts} system as a tool for temporally regulating gene expression. Another genetic tool for the control of gene expression is the Gene-Switch system (McGuire *et al.* 2004), which would avoid the problems associated with temperature effects on behavior. However, because this system relies on drug treatment to induce gene expression, we would be unable to use it to assess expression requirement during the pupal period. However, this system might be useful for better assessing the physiological requirements of normalized insulin signaling for memory.

Another interesting question to consider is whether *dfmr1* expression in the IPCs also has similar temporal requirements to that of insulin signaling. We could perform this experiment by initiating *dfmr1* expression at the beginning of the pupal stage or in adulthood and examining the expression parameters that affected circadian behavior and memory. If the temporal requirements for *dfmr1* expression coincided with the requirements for insulin signaling normalization, it would suggest that the presence of *dfmr1* in the IPCs was important for normal release of insulin in response to external stimuli. Conversely if the temporal requirements did not overlap, it would suggest that *dfmr1* was required for a process that indirectly leads to defective release of insulin at a later time point.

Our final experiments examined the temporal requirements of metformin treatment for circadian behavior and memory. However, we were unable to rescue circadian behavior with metformin treatment administered in development, adulthood, or at both times. These results suggest that while memory is amenable to rescue by metformin treatment, circadian behavior is not. However, we must consider that metformin treatment may not have rescued circadian behavior because metformin treatment, like insulin signaling normalization, is required during the pupal period. Since flies do not consume food while they are undergoing metamorphosis during the pupal stage, it is extremely difficult to treat them with drugs during this period. However, it is not altogether unprecedented to treat pupae with drugs (GATTO AND BROADIE 2009), though the process is extremely tedious. This method might allow us to address the question of whether metformin is unable to rescue the circadian defect, or whether poor timing of metformin administration led to the lack of rescue.

In this chapter, we identified the pupal stage as being critical for the proper development of circadian behavior in response to insulin signaling. Furthermore, we determined that metformin treatment does not rescue circadian behavior, but can rescue STM in the conditioned courtship paradigm when given in either development or adulthood. Finally, we showed that while metformin effectively rescues olfactory-based memory when administered during adulthood, administration during development does not rescue memory. Altogether, these findings indicate that although these three behaviors are all affected by insulin signaling misregulation in *dfmr1* mutants, moderation of insulin signaling is required at different time points for each of them to restore normal behavior.

Figures:

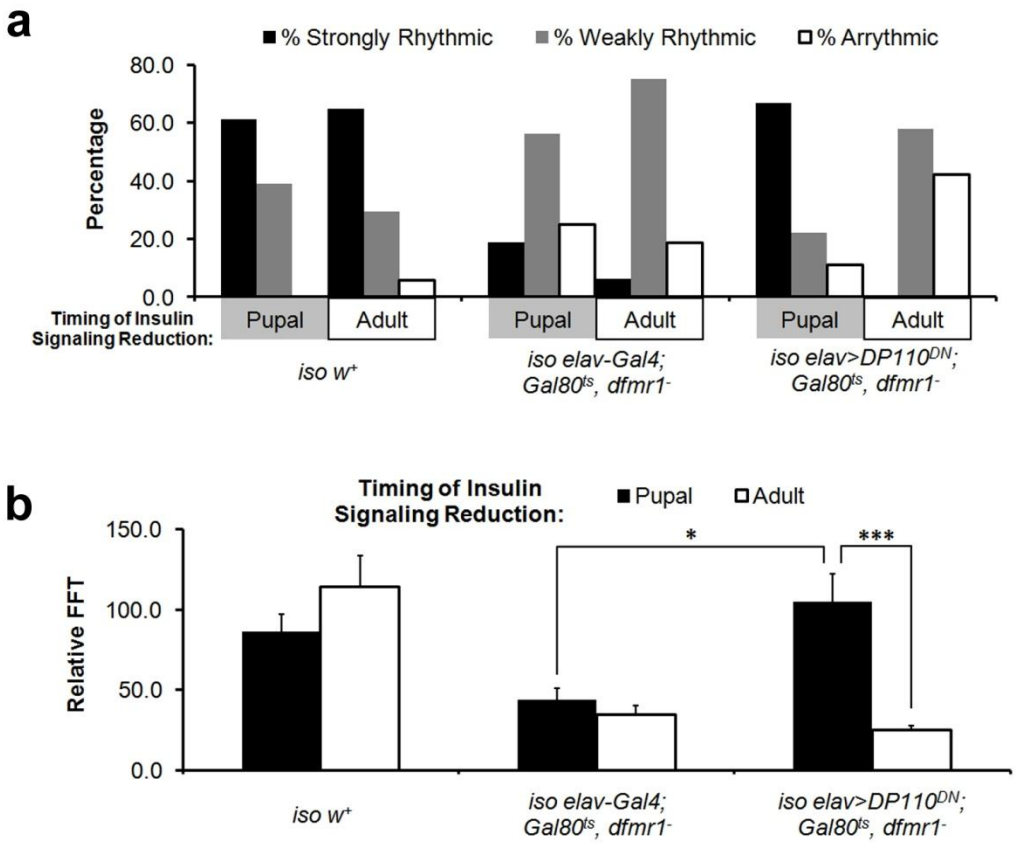

Figure 4-1. A pan-neuronal decrease in PI3K activity must occur during the pupal period to rescue circadian rhythmicity. The percentages of strongly rhythmic (FFT≥0.04), weakly rhythmic (0.04>FFT≥0.01) and arrhythmic flies (FFT<0.01) are shown. *Dfmr1* mutant flies pan-neuronally expressing $DP110^{DN}$ beginning in the pupal period show **(a)** a greater percentage of strongly rhythmic flies and **(b)** significantly improved rhythmicity compared to flies in which $DP110^{DN}$ was expressed starting in adulthood, ($p<0.001$), and compared to $dfmr1^{-}$ controls *(elav-Gal4; $Gal80^{ts}$,$dfmr1^{-}$)* also shifted to 29°C at the start of the pupal period, ($p<0.05$). N=16-19. Statistical significance is denoted with asterisks (*$p<0.05$, ** $p<0.01$, ***$p<0.001$). Statistical significance was determined by a Kruskal-Wallis test and Dunn's post-test. Graphs depict mean ± s.e.m.

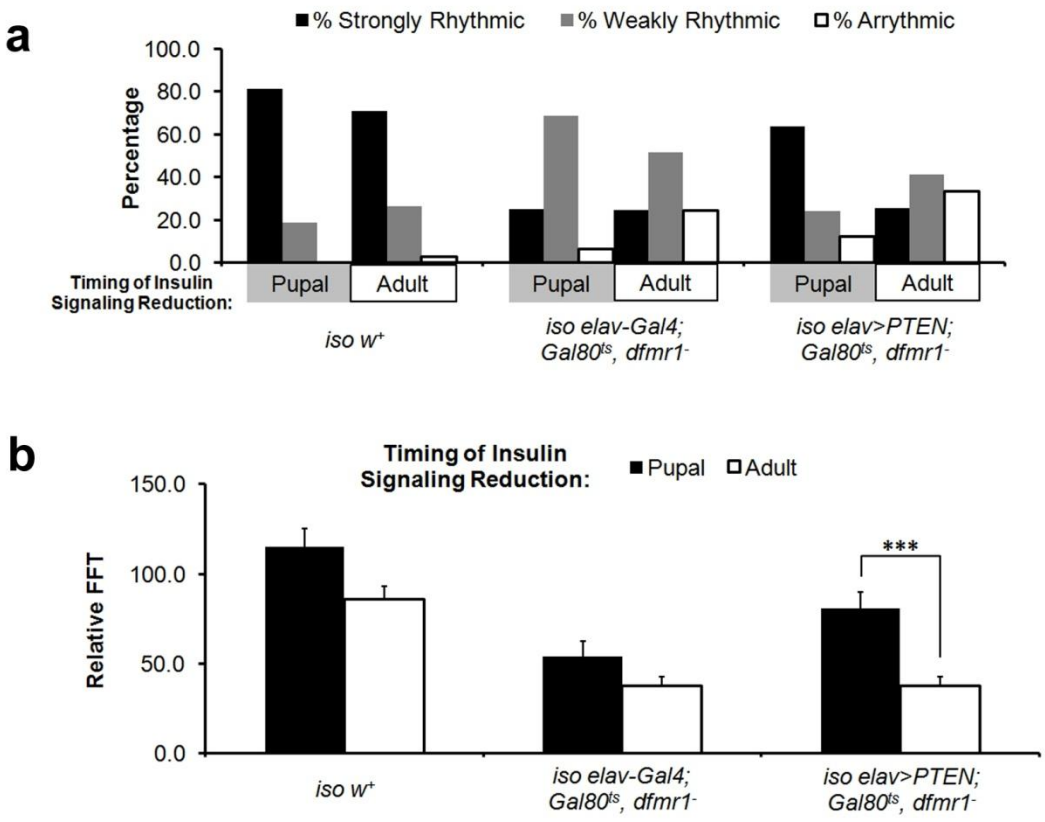

Figure 4-2. Expression of PTEN during the pupal stage improves circadian rhythmicity in *dfmr1* mutants. The percentages of strongly rhythmic (FFT≥0.04), weakly rhythmic (0.04>FFT≥0.01) and arrhythmic flies (FFT<0.01) are shown. *Dfmr1* mutants pan-neuronally over-expressing PTEN starting at the beginning of the pupal period show **(a)** an increased percentage of strongly rhythmic flies and **(b)** significantly better rhythmicity than flies with PTEN expression initiating in adulthood ($p<0.001$) as well as a trend towards increased rhythmicity compared to *dfmr1* mutant controls also shifted to 29°C starting in the pupal period. N=16-39. Statistical significance is denoted with asterisks (*$p<0.05$, ** $p<0.01$, ***$p<0.001$). Statistical significance was determined by a Kruskal-Wallis test and Dunn's post-test. Graphs depict mean ± s.e.m.

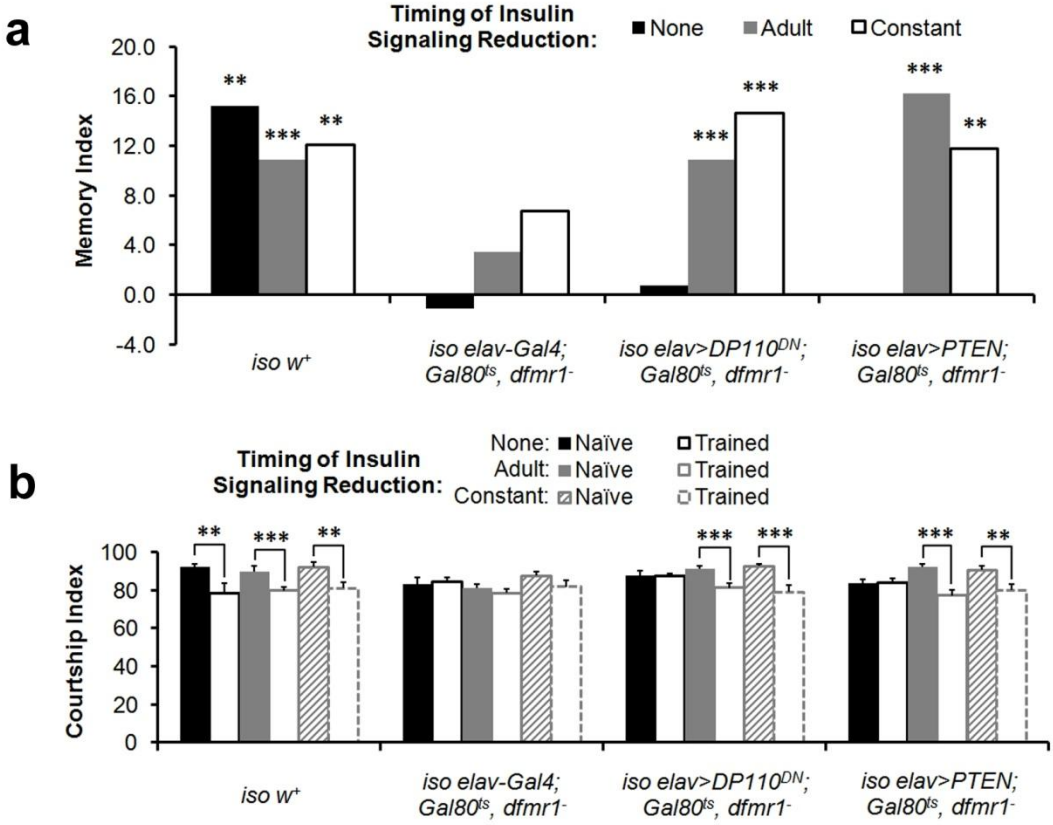

Figure 4-3. Reduction of insulin signaling in adulthood is sufficient to restore STM in *dfmr1* mutants. STM in the courtship paradigm is presented as **(a)** memory index (MI), which conveys the difference between the courtship indices (CIs) of trained and untrained flies in a single value (MI = $(CI_{naive}-CI_{trained})/CI_{naive}$), and **(b)** as separate CIs. If memory is present, the CI of trained flies will be reduced compared to the CI of naïve flies, indicating that the trained fly remembers the negative experience of rejection by an unreceptive female. Reduction of insulin signaling starting in adulthood (using either pan-neuronal expression of $DP110^{DN}$ or PTEN) rescues STM in *dfmr1* mutants. N=16-76. Data were subject to an arcsine transformation to obtain a normal distribution, and then statistical significance was determined by ANOVA with pre-planned comparisons. Statistical significance is denoted with asterisks (*p<0.05, ** p<0.01, ***p<0.001). Graphs depict mean ± s.e.m. These data were obtained in collaboration with Sean McBride.

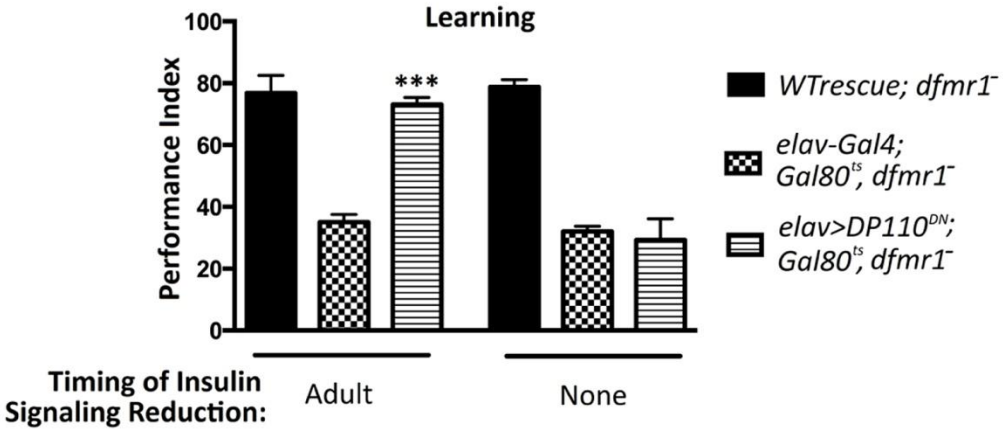

Figure 4-4. Decreasing PI3K activity in adulthood is sufficient to restore learning to *dfmr1* mutants in the olfactory conditioning assay. Performance index (PI) represents the percent of flies which avoid the shock-conditioned odor. (PI = % of flies avoiding shock-conditioned odor - % of flies moving toward shock conditioned odor). *Dfmr1* mutant flies with pan-neuronal adulthood expression of $DP110^{DN}$ show rescue of the olfactory-based learning defects (N=4, p=0.0001). Statistical significance was determined by an ANOVA with Bonferroni correction and a Tukey post-test. Statistical significance is denoted with asterisks (*p<0.05, ** p<0.01, ***p<0.001). Graphs depict mean ± s.e.m. These data were obtained by Daniel Chambers and Francois Bolduc.

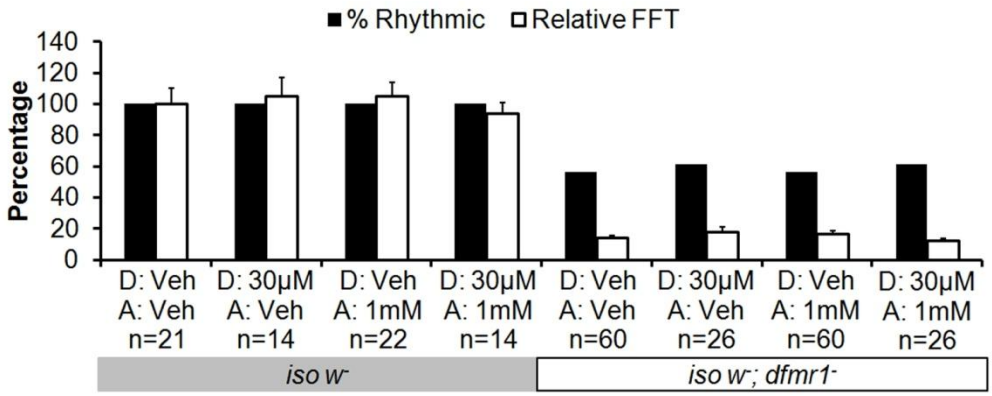

Figure 4-5. A 30μM developmental and 1mM adulthood metformin treatment regime does not rescue circadian behavior in *dfmr1* mutants. The circadian behavior of flies raised on 30μM metformin and moved to 1mM metformin or vehicle control food within 24 hour of eclosion was examined. Metformin treatment does not improve the rhythmicity of *dfmr1* mutants. Rhythmicity was determined by FFT (.01 or higher) and visual inspection of the actogram and periodogram. Statistical significance is denoted with asterisks (*p<0.05, ** p<0.01, ***p<0.001). Statistical significance was determined by a Kruskal-Wallis test and Dunn's post-test. Graphs depict mean ± s.e.m.

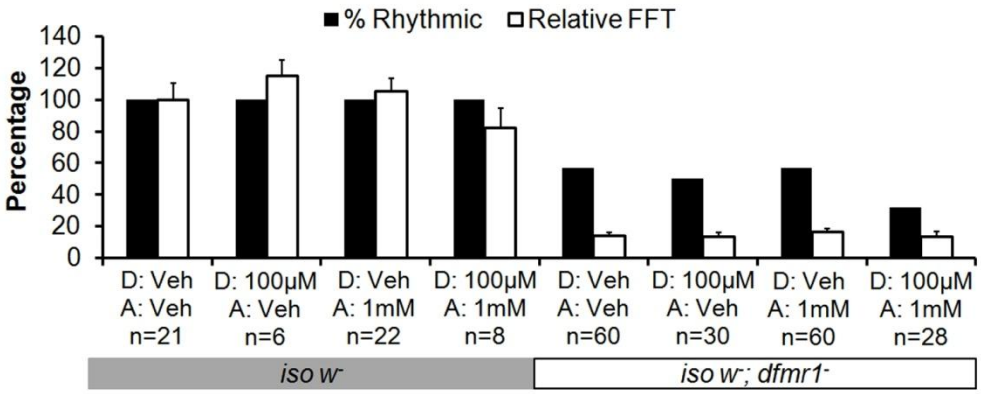

Figure 4-6. A 100μM developmental and 1mM adulthood metformin treatment regime does not rescue circadian behavior in *dfmr1* mutants. The circadian behavior of flies raised on 100μM metformin and moved to 1mM metformin or vehicle control food within 24 hour of eclosion was examined. Metformin treatment does not improve the rhythmicity of *dfmr1* mutants. Rhythmicity was determined by FFT (.01 or higher) and visual inspection of the actogram and periodogram. Statistical significance is denoted with asterisks (*p<0.05, ** p<0.01, ***p<0.001). Statistical significance was determined by a Kruskal-Wallis test and Dunn's post-test. Graphs depict mean ± s.e.m.

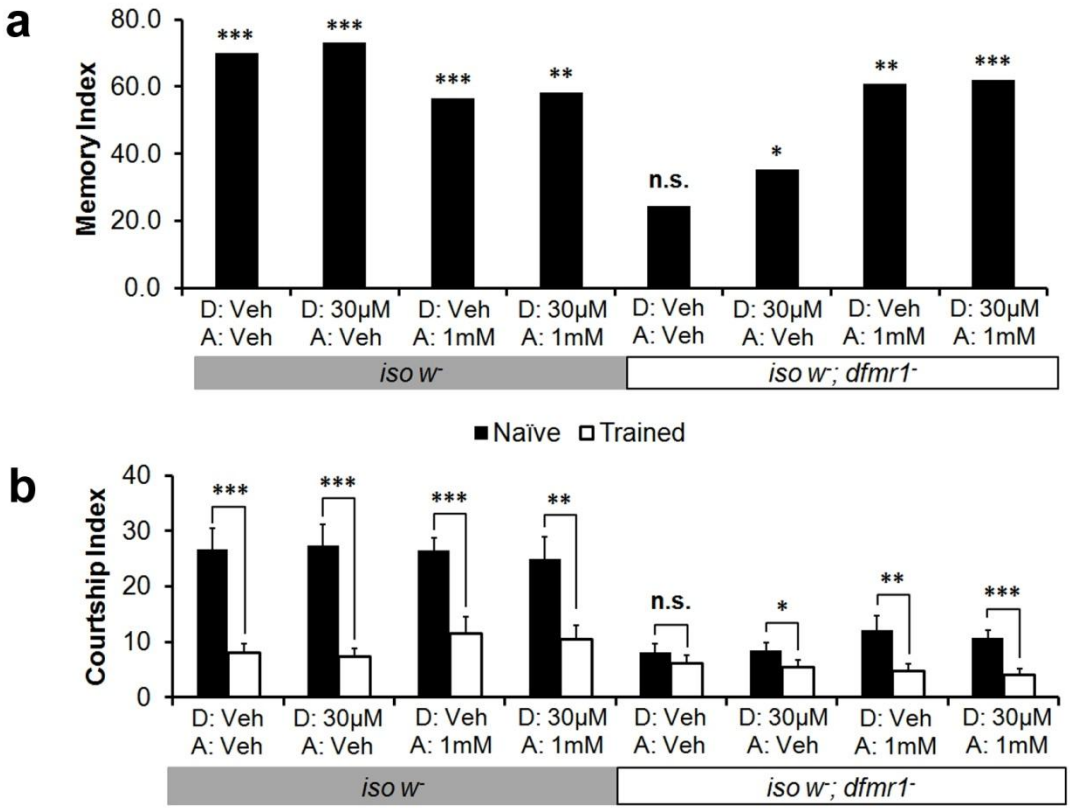

Figure 4-7. A 30μM developmental and 1mM adulthood metformin treatment regime rescues the STM defect of *dfmr1* mutants. Flies raised on 30μM metformin and moved to 1mM metformin or vehicle control food within 24 hours of eclosion were tested in the conditioned courtship paradigm. Treatment with either 30μM or 100μM metformin in development alone, or paired with 1mM metformin treatment in adulthood rescues STM in *dfmr1* mutant flies. Treatment with 1mM metformin in adulthood alone also rescues STM in *dfmr1* mutants. STM in the courtship paradigm is presented as **(a)** memory index (MI), which conveys the difference between the courtship indices (CIs) of trained and untrained flies in a single value ($MI = (CI_{naive}-CI_{trained})/CI_{naive}$), and **(b)** as separate CIs. If memory is present, the CI of trained flies will be reduced compared to the CI of naïve flies, indicating that the trained fly remembers the negative experience of rejection by an unreceptive female. Data were subject to an arcsine transformation to obtain a normal distribution, then statistical significance was determined by ANOVA with pre-planned comparisons. Statistical significance is denoted with asterisks (*p<0.05, ** p<0.01, ***p<0.001). N ranges from 17-27. These data were obtained in collaboration with Sean McBride.

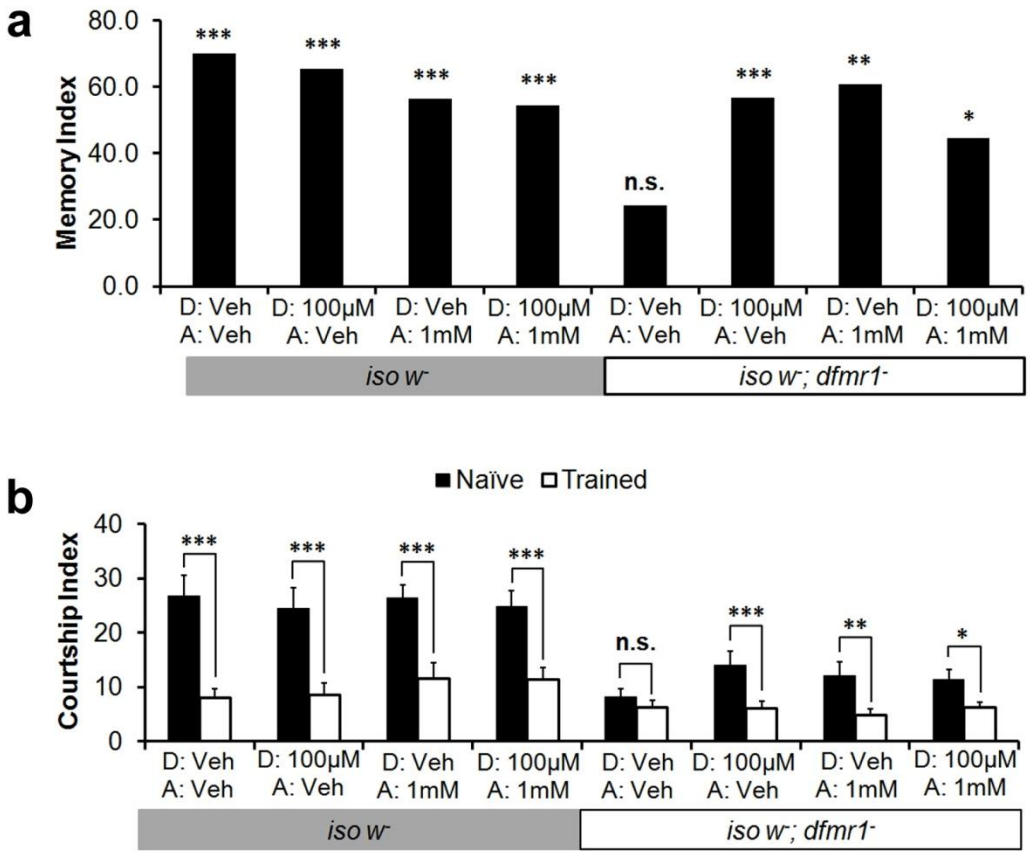

Figure 4-8. A 100μM developmental and 1mM adulthood metformin treatment regime rescues the STM defect of *dfmr1* mutants. Flies raised on 100 μM metformin and moved to 1mM metformin or vehicle control food within 24 hours of eclosion were tested in the conditioned courtship paradigm. Treatment with either 30μM or 100μM metformin in development alone, or paired with 1mM metformin treatment in adulthood rescues STM in *dfmr1* mutant flies. Treatment with 1mM metformin in adulthood alone also rescues STM in *dfmr1* mutants. STM in the courtship paradigm is presented as **(a)** memory index (MI), which conveys the difference between the courtship indices (CIs) of trained and untrained flies in a single value ($MI = (CI_{naive}-CI_{trained})/CI_{naive}$), and **(b)** as separate CIs. If memory is present, the CI of trained flies will be reduced compared to the CI of naïve flies, indicating that the trained fly remembers the negative experience of rejection by an unreceptive female. Data were subject to an arcsine transformation to obtain a normal distribution, and then statistical significance was determined by ANOVA with pre-planned comparisons. Statistical significance is denoted with asterisks (*p<0.05, ** p<0.01, ***p<0.001). N ranges from 17-27. These data were obtained in collaboration with Sean McBride.

Materials and Methods:

Fly stocks and maintenance

Fly stocks were maintained on standard cornmeal-molasses medium. All food used in the circadian and conditioned courtship paradigm experiments was made from the lab recipe. Fly stocks carrying the *UAS-DP110^{DN}* and *UAS-PTEN* transgenes were obtained from M. Birnbaum (LEEVERS *et al.* 1996; HUANG *et al.* 1999). The *elav-Gal4* transgene was derived from Bloomington Stock number 8765. The *dfmr1^{3}*allele and *WTrescue* are previously described in (DOCKENDORFF *et al.* 2002). The *Gal80^{ts}*lines were derived from Bloomington Stock #7018. Flies used were outcrossed to *w1118(iso31B)* flies.

Circadian Behavior Assay

Flies intended for circadian rhythmicity analysis were raised at 25°C on a L:D cycle. Male flies were collected at 0-3 days of age and maintained on standard fly food in an L:D incubator for entrainment. After 3-5 days, individual flies were loaded into 2% agar, 5% sucrose tubes, which were subsequently placed in activity monitors (TriKinetics) and maintained in dark:dark conditions for 10 days.

Data were collected in 5 minute bins and analyzed with Clock Lab software (Actimetrics) to obtain period and rhythmicity values. Rhythmicity was determined by fast fourier transform (FFT) analysis (with rhythmicity defined as a FFT value of 0.01 or more) as well as visual inspection of the actogram and periodogram. Significant differences in average FFT values between genotypes were determined using a Kruskal-Wallis test followed by a Dunn's post-test (GraphPad, InStat). Relative FFT was calculating by dividing the average FFT value of the depicted genotype by the average FFT value of the wild-type control: Relative FFT = $FFT_{depicted}/FFT_{wild\text{-}type}*100$.

For metformin treatment, flies were raised on food containing 30µM or 100µM metformin or vehicle (water). Within 24 hours of eclosion, adult males were collected and maintained on food containing 1mM metformin or vehicle.

For temperature shift experiments, flies with *Gal80^{ts}* were raised at 18°C. White 0-hour pupae or adults were collected while at 18°C and moved to 29°C for 2 days, then moved to 25°C to reduce deleterious effects caused by high temperature. Circadian behavior was tested at 25°C. Flies raised constantly at 18°C or 29°C had reduced circadian rhythmicity and could not be used as controls for these experiments.

Short-term memory assay

Virgin male flies were collected under CO_2 anesthesia every four hours and maintained on standard fly food in small all-male groups at 25°C in L:D until testing. Virgin X^X, *yf* test females were collected on the day of eclosion and kept in food vials. Flies were aged in a 12hr:12hr LD incubator before behavioral training and testing was performed during the relative light phase. All male subjects were transferred to fresh control food the day before testing and assigned to random groups for behavioral training and testing. All training and testing was performed blind to genotype and treatment. A courtship index (CI) was calculated following testing as the percentage of total observation time spent courting. CIs of tested males were subjected to arcsin square root transformations to approximate normal distributions. ANOVAs were performed on pre-planned pair-wise comparisons of arcsin transformed data to get critical p-values.

For data not normal after transformation, the Mann-Whitney test was used to generate p-values (SIEGEL 1957). Statistics were performed using Statview 3.0 and Prism. Memory Index (MI) = $(CI_{naive}-CI_{trained})/CI_{naive}$ (KELEMAN *et al.* 2007).

For drug treatment, flies were raised on food containing 30µM or 100µM metformin or vehicle (water). Adult male flies were collected within 4 hours of eclosion and placed on food

containing 1mM metformin or vehicle (water). One day before STM testing flies were moved to individual vials containing standard fly food.

For temperature shift assays, flies with *Gal80^{ts}* were raised at 22°C, then moved to 27°C within 4 hours of eclosion. (22°C was used as the permissive temperature due to the deleterious effects of 18°C on courtship). All flies were moved to 25°C the night prior to testing and were tested at 25°C.

Pavlovian olfactory learning and memory

Flies were raised at 22^{o}C and placed at 25^{o}C overnight prior to behavioral experiments. Adult *Drosophila* 1-3 days old were trained and tested with the classical conditioning procedure.

About 100 flies were trapped inside a training chamber covered with an electrifiable copper grid. Flies were allowed 90 seconds to acclimate and then were exposed sequentially to two odors, 3-octanol (OCT) and 4- methylcyclohexanol (MCH), carried through the chamber in a current of air. Relative concentrations of OCT and MCH were adjusted so that naïve flies distributed themselves 50:50 in the T-maze. Flies first were exposed for 60 seconds to the conditioned stimulus (CS+; either OCT or MCH, depending on the odor the flies were shocked to in the first step), during which time they received the unconditioned stimulus (US; twelve 1.25 seconds pulses of 60V DC electric shock at 5 second interpulse intervals). After the CS+ presentation, the chamber was flushed with fresh air for 45 seconds. Then flies were exposed for 60 seconds to a second, control stimulus (CS-; either MCH or OCT), which was not paired with electric shock. After the CS- presentation, the chamber was again flushed with fresh air for 45 seconds.

To test for conditioned odor avoidance after classical conditioning, flies were moved to the choice point of the T-maze. Ninety seconds later, the flies were exposed to two converging current of air one carrying OCT, the other MCH, from opposite arms of the T-maze. Flies were allowed to choose between the CS+ and CS- for 120 seconds, at which time they were trapped inside their respective arms of the T-maze, anesthetized and counted.

For long-term memory testing, 1-3 days old adult flies were subjected to classical (Pavlovian) olfactory conditioning for 10 training sessions without a rest interval (massed training) or to 10 training sessions with 15 minutes rest between each training session (spaced training). After training, flies were stored at 18^{o}C and then conditioned responses were tested after a 24-hour retention interval at 25°C.

For both learning and long-term memory, two groups of flies were trained and tested in one complete experiment. The CS+ was OCT and the CS- was MCH for one group; the CS+ was MCH and the CS- was OCT for the second group. The performance index (PI) was calculated as the average of the fraction of the population avoiding the shock-associated odor minus the fraction avoiding the control odor for each group of flies trained in one experiment. In other words, the PI enumerates the distribution of flies in the T-maze as a normalized “percent correctly avoiding the shock-paired odor” and ranges from 0 for a 50:50 distribution to 100 for a 100:0 distribution.

Data from an experiment were subjected to a one-way ANOVA (JMP from SAS, Inc.), followed by planned pair-wise comparisons. An alpha = 0.05 was corrected for multiple comparisons using Bonferroni. Post-test analysis was performed with the Tukey test.

For drug treatment, flies were placed in vials overnight at 25°C and 70% humidity with Whatman filter paper containing 200 uL of either 1mM metformin or vehicle and 5% sucrose.

For temperature shift experiments, flies were raised at 18°C, moved to 27°C after eclosion and incubated for 4 days prior to testing learning.

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Chapter 5: Discussion and Conclusions

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Abstract:

In the previous chapters, we showed that insulin signaling is disrupted in the *dfmr1* mutant fly model of fragile X syndrome (FXS), and that this misregulation contributes to behavioral abnormalities in the mutant fly. We found that the behavioral abnormalities could be rescued by genetic and sometimes pharmacological means of reducing insulin signaling. Critically, we also presented evidence that insulin normalization during the pupal stage is important for circadian behavior, but not for rescue of memory.

Although these findings reveal a new pathway involved in FXS, they leave us with many new questions as well. We still need to determine the mechanism by which dFMR1 functions in the IPCs to promote normal insulin signaling in *Drosophila.* We also need to determine the mechanisms by which insulin signaling affects circadian behavior and memory in *dfmr1* mutants. Furthermore, it will be important to further investigate the developmental role of insulin signaling in the pupal period to better understand how its misregulation leads to aberrant circadian behavior. Finally, we need to investigate the mechanisms by which metformin acts to restore memory in *dfmr1* mutants, and determine if it would be a useful pharmacological treatment in the *Fmr1* KO mouse, and potentially in humans.

Introduction:

Fragile X syndrome (FXS) is serious illness affecting many individuals across the world (TURK 2011). Because it is a major cause of autism and intellectual disability (JACQUEMONT *et al.* 2007), revealing new signaling pathways disrupted in FXS will heighten our understanding of the signaling mechanisms underlying other forms of cognitive and social disabilities. Using an unbiased approach, we uncovered a connection between altered insulin signaling and FXS. Our findings raise several interesting questions about the mechanism by which dFMR1 influences insulin signaling, and whether altered insulin signaling contributes to behavior phenotypes in the mouse FXS models or to cognitive and/or sleep problems in human patients.

We have shown that the IPCs are involved in the circadian output pathway, and that normal dFMR1 expression in these cells is important for normal memory and circadian rhythmicity. We also showed that insulin signaling is increased in the brains of *dfmr1* mutant flies, and that expression of *dfmr1* in the IPCs of *dfmr1* mutants rescues this increase in a non-cell autonomous manner. Furthermore, reducing insulin signaling genetically resolves the memory and circadian defects in *dfmr1* mutants, suggesting that misregulated insulin signaling in *dfmr1* mutants contributes to the abnormal behavior of these flies. We further showed that to rescue circadian behavior, reduction of insulin signaling has to be present during the pupal period, whereas to rescue memory, the reduction does not have to be present until adulthood. Finally, we showed that treatment with the Type II diabetes drug metformin was able to rescue memory.

The identification of this new signaling pathway connected to FXS raises many questions. We do not know how dFMR1 acts in the IPCs to regulate insulin signaling, nor do we know the how this discovery would translate to humans. Our data also provide evidence that abnormal insulin signaling is a factor in the memory and circadian deficits seen in *dfmr1* mutants, raising the question of how dysregulation of insulin signaling could cause behavioral problems. Finally, our discovery of metformin as possible treatment for cognitive abnormalities in FXS presents the possibility of a new target for pharmacological treatment, especially if we can identify its mechanism of action.

Possible mechanisms of dFMR1 action in IPCs:

In Chapter 2, we showed that insulin signaling is perturbed in *dfmr1* mutant flies. Although dFMR1 is expressed throughout the nervous system, we made the intriguing discovery that directing *dfmr1*expression to a small number of neurons, the IPCs, could rescue two major behavioral phenotypes of the *dfmr1* mutant fly. As referenced previously, the IPCs are a group of 14 cells in the PI region of the brain. They are part of a larger population of neuropeptide producing cells known as the median neurosecretory cells, which is thought to be analogous to the mammalian hypothalamus (NASSEL *et al.* 2013).

One major question that must be addressed is the mechanism by which dFMR1 functions in the IPCs to ensure normal insulin signaling. Our results tell us that expression of *dfmr1* in the IPCs affects insulin signaling in a cell non-autonomous manner. Specifically, we observed that expressing *dfmr1* in the IPCs was sufficient to restore pAkt levels to normal in other parts of the brain, indicating that the action of dFMR1 in the IPCs resulted in a signal that affected other cells. These results suggest that the presence of dFMR1 in the IPCs affects their ability to release insulin into the environment. We then must ask how dFMR1 could affect the insulin production and release of the IPCs.

Since dFMR1 is an RNA-binding protein which primarily represses the translation of its targets (SANTORO *et al.* 2012), one possibility is that dFMR1 directly binds to *dilp2* mRNA and represses its translation. In the absence of dFMR1, *dilp2* translation, and thus Dilp2 protein levels, would increase. This hypothesis is consistent with our findings that Dilp2 protein levels are increased in the IPCs while *dilp2* mRNA levels remain unchanged. The increase in Dilp2 levels could then result in an increased amount of Dilp2 released when the IPCs were activated.

A second possible mechanism is that dFMR1 acts more broadly within the IPCs to promote normal synaptic architecture and synapse formation. Importantly, loss of FMR1 in neurons is known to cause morphological defects in *Drosophila,* mice and humans (ZHANG *et al.* 2001; DOCKENDORFF *et al.* 2002; MORALES *et al.* 2002; O'DONNELL AND WARREN 2002; REEVE *et al.* 2005; JACQUEMONT *et al.* 2007). If *dfmr1* was important for morphology of the IPCs, loss of

dfmr1 could potentially result in neurons that were unable to effectively receive signals from presynaptic cells.

Alterations in functional synapses between the IPCs and their neighboring neurons would likely have serious implications for insulin signaling. Since the IPCs are part of a major group of neurosecretory cells, they are the recipient of signals from many neural groups. Neurons expressing Serotonin, *Drosophila* tachykinin, GABA, corazoin, and small neuropeptide F (sNPF) impinge on the IPCs and regulate facets of insulin signaling and metabolism (ENELL *et al.* 2010; BIRSE *et al.* 2011; KAPAN *et al.* 2012; LUO *et al.* 2012). Perhaps the most well understood of these circuits is that of the GABAergic neurons. These neurons synapse with the IPCs and inhibit secretion of Dilps until the fat body-derived leptin homolog, Unpaired2, signals the GABAergic cells to relieve this inhibition (RAJAN AND PERRIMON 2012). If lack of *dfmr1* in the IPCs resulted in reduced synapsing with the GABAergic neurons, the break on insulin signaling would be lessened, potentially leading to increased overall insulin signaling.

Although reduction of connections with the GABAergic neurons would cause the most straightforward disruption of insulin signaling from the IPCs, changes in synapse number between any of the aforementioned neurons and the IPCs would results in changes in metabolism. Interruption of these connections causes metabolic defects such as changes in lifespan, a classic hallmark of insulin signaling, as well as alterations in glucose, trehalose, glycogen and lipids (BIRSE *et al.* 2011; KAPAN *et al.* 2012; LUO *et al.* 2012). In one case, different neuropeptides produced by the same neurons effect on metabolism in different ways (KAPAN *et al.* 2012). To further complicate interpretation of signaling to the IPCs, it is possible that dendrites and release sites in these cells are close together (NASSEL *et al.* 2013), indicating that it could be difficult to know if a particular neural group signals to the IPCs or receives signals from the IPCs.

We should also consider the possibility that dysregulation of signaling in the IPCs caused by loss of *dfmr1* might affect pathways beyond insulin signaling as it has been noted that some neuropeptides signal through the IPCs for purposes other than regulation of insulin signaling. Most notably, octopaminergic neurons activate the IPCs to promote wakefulness, but this function

is independent of insulin signaling (CROCKER *et al.* 2010; ERION *et al.* 2012). Furthermore, insulin seems to regulate other behaviors in conjunction with the IPCs and their neighboring neurons. sNPF expressing neurons, and Drosulfakinins expressed in the IPCs themselves, regulate the willingness of flies to eat non-palpable foods (WU *et al.* 2005; SODERBERG *et al.* 2012). These results suggest that if the morphology of the IPCs is abnormal, other behaviors, such as sleep and feeding could be disrupted. Indeed, *dfmr1* mutants have already been noted to have abnormal sleep behavior (BUSHEY *et al.* 2009; VAN ALPHEN *et al.* 2013).

Further experiments will need to occur to determine whether *dfmr1* direct interacts with *dilp* transcripts, or whether it regulates insulin signaling from the IPCs more indirectly by affecting signaling inputs and outputs from the cells. Nevertheless, our data suggest that *dfmr1* expression in the IPCs is crucial for normal insulin release and insulin signaling in the *Drosophila* brain.

The finding that *dfmr1* plays an important role in the IPCs raises the question of how this discovery translates to human patients. Curiously, the mammalian cells most analogous to the IPCs are the insulin-producing β-cells in the pancreas (RULIFSON *et al.* 2002). FMR1 is expressed in β-cells, but its function in these cells is not established (MILOCHAU *et al.* 2014). Interestingly, the pancreatic β-cells have many things in common with neurons. They express receptors for multiple neurotransmitters, and can release both glutamate and GABA (OTTER AND LAMMERT 2016). Interestingly, GABA is released from β-cells in parallel with insulin (GYLFE AND TENGHOLM 2014). However, the dense core granules where insulin is stored are several times larger than synaptic vesicles found in neurons (OTTER AND LAMMERT 2016). Similarly to neurons, neurotransmitters regulate the release of insulin from the β-cells. Serotonin and dopamine both suppress insulin release through a negative feedback loop (GYLFE AND TENGHOLM 2014). However, the ultimate signal to release insulin does not come from neurons, but rather through neurotransmitters released by the islet cells (OTTER AND LAMMERT 2016). Clearly, despite their different locations, β-cells share striking similarities to the *Drosophila* IPCs and neurons in general, raising the possibility that FMR1 may have an important role in β-cells that is worth further exploration.

There are several interesting facets of β-cell biology that suggest a possible role for FMR1 which may provide further clues as to how dFMR1 functions in the IPCs. First, mGluRs are among the factors that have an important role in insulin secretion. As was discussed in Chapter 1, the mGluR pathway is one of the major pathways thought to be disrupted in FXS (BEAR *et al.* 2004). mGluR is necessary for well-regulated release of insulin from β-cells, and has been shown to have both excitatory and inhibitory roles in release (STORTO *et al.* 2006; OTTER AND LAMMERT 2016). It is possible that mGluR signaling is also important for Dilp release from the IPCs, raising the possibility that abnormal mGluR signaling caused by loss of *dfmr1* in the IPCs could cause altered insulin secretion in *dfmr1* mutants. A second possible mechanism of FMR1 function in through the miRNA pathway. It has been well established that FMR1 is a component of the RISC complex and can use miRNAs to suppress translation (CAUDY *et al.* 2002; JIN *et al.* 2004; EDBAUER *et al.* 2010). Crucially, β-cells require miRNAs for normal function (RUTTER *et al.* 2015), and miRNAs have been shown to play an important role in the IPCs as well (VARGHESE *et al.* 2010). The miRNA pathway is yet another potential mechanism through which FMR1 could regulate insulin signaling in the β-cells and IPCs.

Our findings suggest that *dfmr1* performs an important role in the IPCs that is closely linked to the ability of these cells to release insulin in a normal manner. Thus it is important to explore the possible mechanisms of dFMR1 function in the IPCs, as well as to determine if FMR1 functions by a similar method to regulate insulin release from β-cells. If loss of FMR1 does cause faulty insulin release from β-cells, it could have interesting implications for metabolism in FXS patients.

Evidence for insulin signaling irregularities in mammals with loss of *FMR1*

In Chapter 2 we showed that insulin signaling in the brains of *dfmr1* mutants is disrupted, and in Chapter 3 we showed that this disruption contributes to circadian arrhythmicity and loss of memory. These results raise the question of whether the loss of insulin signaling regulation is conserved in other FXS models and human patients. Although the insulin pathway has not been

actively investigated in *Fmr1* knockout mice or FXS patients, some hints exist that misregulation of insulin signaling is conserved in mammals. Indeed, data from the mouse model have identified elevated signaling in the PI3K-Akt-mTOR pathway that lies downstream of the insulin receptor (GROSS *et al.* 2010; SHARMA *et al.* 2010). Also, weight of *FMR1* KO mice is increased in early development relative to littermate controls, a finding that is also consistent with increased insulin signaling, at least in very young mutant pups (DOLEN *et al.* 2007). Interestingly, some observations of FXS patients hint that insulin signaling may be abnormal. A subgroup of patients exhibits a distinctive obese phenotype (NOWICKI *et al.* 2007) yet FXS patients have been observed to have decreased cholesterol (Berry-Kravis et al. 2014), suggesting that loss of *Fmr1* leads to metabolic abnormalities. Furthermore, a more recent study identified markers of elevated insulin signaling in the blood and brains of FXS patients (HOEFFER *et al.* 2012). These observations could indicate that elevated insulin signaling is a conserved characteristic in the mouse FXS model and possibly FXS patients and that the full insulin signaling profile of FXS patients and the *Fmr1* KO deserves further examination.

Evidence for the importance of insulin signaling in the brain

Our finding that altered insulin signaling contributes to circadian and memory defects in *dfmr1* mutant flies raises the question of how insulin could affect behavior. Although insulin signaling is most known for promoting growth and was once thought to be unimportant in the brain since insulin is not required for glucose uptake in neurons (HENI *et al.* 2015), it has been revealed to have an important role in both brain development and maintenance. Insulin signaling via either insulin or Insulin-like Growth Factor 1 (IGF1) or Insulin-like Growth Factor 2 (IGF2) during development is critical for neural differentiation, proliferation, axon development and synapse formation (FERNANDEZ AND TORRES-ALEMAN 2012). Critically, reduced insulin signaling during development results in microcephaly (FERNANDEZ AND TORRES-ALEMAN 2012). In keeping with the critical role of insulin signaling in the brain, the insulin receptor is expressed throughout the brain with high concentrations in the hippocampus, an area important for learning and

memory (DE FELICE AND BENEDICT 2015; KIM AND FELDMAN 2015). In mammals, insulin has not been shown to be produced in the brains of adults, so insulin must enter the brain either through the blood-brain barrier, or the choroid plexus (FERNANDEZ AND TORRES-ALEMAN 2012; GRAY *et al.* 2014). The importance of insulin signaling in the brains of mammals confirms that the possible for insulin signaling in cognition and circadian rhythmicity in *dfmr1* mutant flies could be conserved in mammals as well.

The differences between brain insulin signaling and peripheral insulin signaling could explain one puzzling aspect of our fly model of FXS: although insulin signaling is increased in *dfmr1* mutant flies, we have not observed that *dfmr1* mutants are larger than wild-type flies. It is consistently reported that in *Drosophila,* increasing insulin signaling results in bigger cells and more numerous cells (LEEVERS *et al.* 1996; BROGIOLO *et al.* 2001; WERZ *et al.* 2009), suggesting that the *dfmr1* mutant phenotype we report is far more complicated than a simple increase in insulin signaling could explain. Interestingly, while insulin signaling in the body generally promotes growth and weight gain in adults, insulin signaling in the brain has the opposite effect, resulting in a general trend toward weight loss (BLAZQUEZ *et al.* 2014; HENI *et al.* 2015). This observation would be consistent with the effects we see in the *dfmr1* mutant fly, and potentially suggests that the insulin signaling increase we see in *dfmr1* mutant flies is not ubiquitous and may be specific to the brain.

Possible roles for insulin signaling in circadian behavior:

Although no role for insulin signaling in circadian behavior has yet been established, several studies hint at roles for insulin signaling in circadian behavior. Changes in insulin signaling affect sleep duration in older flies (YURGEL *et al.* 2015), which could also affect rhythmicity. It is also known that *dfmr1* mutant flies have sleep defects (BUSHEY *et al.* 2009; VAN ALPHEN *et al.* 2013). Furthermore, alterations in ecydsone signaling are known to affect circadian rhythmicity (ITOH *et al.* 2011; KUMAR *et al.* 2014). Interaction between the ecydsone and insulin pathways is involved in adult size determination, and ecydsone is essential for control of insulin

signaling during the pupal period (COLOMBANI *et al.* 2005; OKAMOTO *et al.* 2009; SLAIDINA *et al.* 2009), so it is feasible that these two pathways also interact to regulate behavior. It is also curious that ecydsone plays an important role during the pupal stage (EGGER *et al.* 2008), a time period we identified as being critical for normal circadian behavior. An interaction between insulin signaling and ecydsone could therefore suggest that insulin signaling's regulation of behavior is intertwined with other signaling pathways.

Necessity of normal insulin signaling for circadian behavior:

Although in Chapter 3 we discovered that reducing insulin signaling was sufficient to rescue circadian rhythmicity in *dfmr1* mutants, we were unable to show that normal insulin signaling was necessary for proper circadian behavior. Specifically, we found that increasing insulin signaling pan-neuronally or in the IPCs in wild-type flies did not recapitulate the circadian deficit of *dfmr1* mutants. It is possible that increased insulin signaling is a contributing factor to circadian misregulation, but is not the single causative factor. Since many signaling pathways have arisen as targets of treatment in FXS (SCHAEFER *et al.* 2015), it is possible that increased insulin signaling causes circadian abnormalities along with one or more of these other pathways. Disruption of both pathways may need to be present to cause arrhythmic circadian behavior such that restoration of balance to just one of these pathways abrogates the circadian defects.

The second possibility is that our insulin signaling manipulations did not accurately recapitulate the insulin signaling environment present in *dfmr1* mutants. To imitate the insulin signaling environment of *dfmr1* mutants, we would have to mimic the magnitude as well as the temporal and spatial qualities of altered insulin signaling in the mutant flies. Perhaps our manipulations did not result in the precise level of insulin signaling needed to cause circadian arrhythmicity. As we found when we tried to increase insulin signaling ubiquitously, high levels of insulin signaling are lethal, and it may be difficult to achieve high insulin signaling without pushing it lethally high. We also do not know the full spatial profile of increased insulin signaling in *dfmr1* mutants. Although we observed insulin signaling to be increased in the brain, we have not

examined whether it is increased throughout the fly. Furthermore, we were unable to achieve a ubiquitous increase in insulin signaling in wild-type flies, a potentially important manipulation if insulin signaling is increased ubiquitously in *dfmr1* mutant flies. Finally, we have not yet examined the temporal profile of insulin signaling in *dfmr1* mutants, so we do not know when insulin signaling is first increased. Increasing insulin signaling too early or too late could explain our failure to replicate circadian arrhythmicity in otherwise wild-type flies. Notably, in Chapter 4, we discovered that insulin signaling must be normalized during the pupal period to rescue rhythmicity in *dfmr1* mutants, indicating that timing of insulin signaling manipulations are critical to their efficacy. Thus it seems plausible that our inability to recapitulate circadian defects in wild-type flies by increasing insulin signaling could be due to a failure to precisely reproduce the insulin signaling defect of *dfmr1* mutants.

Possible roles for insulin signaling in memory:

In Chapter 3 we reported that increased insulin signaling in the brains of *dfmr1* mutants contributes to defects in courtship-based and olfactory-based memory. This finding raises the question of how misregulation of insulin signaling could cause defects in memory. A role of insulin signaling in memory has been established in recent years. The presence of insulin receptors in the hippocampus (DE FELICE AND BENEDICT 2015; KIM AND FELDMAN 2015), a brain region important for learning and memory, hints at a possible role for insulin signaling in cognitive function. Furthermore, insulin signaling is important for synaptic plasticity (FERNANDEZ AND TORRES-ALEMAN 2012). Further hinting at a role for insulin signaling in memory, when rats were examined after training in the Morris water maze, insulin receptor expression and activation was increased (ZHAO *et al.* 1999). Treating the brains of rats with insulin has been shown to have positive effects on their spatial memory (KIM AND FELDMAN 2015). Indeed, intranasal insulin treatment has also been shown to have positive effects on working memory in healthy individuals (KIM AND FELDMAN 2015). Intranasal insulin treatment goes directly to the brain along the optic

nerve, bypassing the blood-brain barrier, so there is no concern about effects of insulin on peripheral tissues (HENI *et al.* 2015).

Perhaps even more interesting is what happens to cognitive function when insulin signaling is perturbed. Knocking out the InR in the nervous system of mice resulted in anxiety, and depressive behaviors (DE FELICE AND BENEDICT 2015), and the IGF-1 mutant mouse has been shown to have deficits in spatial learning (FERNANDEZ AND TORRES-ALEMAN 2012). It appears that insulin resistance in the body may impair transport of insulin into the brain (KAIYALA *et al.* 2000), leading to memory problems. Illustrating this point, rats and fed high fat diets show evidence of cognitive deficits (KIM AND FELDMAN 2015). Indeed, it seems that whole-body insulin resistance may impair transport of insulin into the brain. Interestingly, patients with Diabetes Type I or Type II show a propensity to cognitive problems which disappear once insulin balance is restored (KIM AND FELDMAN 2015). Alzheimer's Disease has also been proposed to be due to insulin resistance in the brain, and has been referred to as "Type 3 Diabetes" (KIM AND FELDMAN 2015). Intriguingly, intranasal insulin treatment is able to improve memory in early-stage Alzheimer's patients (KIM AND FELDMAN 2015). Although our understanding of how insulin signaling affects memory is still in its infancy, our finding that misregulation of insulin signaling contributes to memory deficits in *dfmr1* mutant flies is in line with the emerging understanding that insulin signaling in the brain is critical for cognition. It should be noted that in many of the instances cited above, increased insulin signaling in the brain had positive effects on memory, in contrast to the our finding that increased whole brain insulin signaling is apparently detrimental for memory. However, the studies examined short-term insulin administration, and it is entirely possible that long-term insulin administration could cause harmful side effects.

Altogether, misregulated insulin signaling could absolutely be a major contributor to the cognitive and circadian deficiencies in *dfmr1* mutant flies. A better understanding of the role of insulin signaling in circadian behavior and memory is critical to our ultimate ability to better treat FXS.

Developmental insulin signaling requirements for circadian behavior and memory:

In Chapter 4, we identified the pupal stage as a critical time period during which insulin signaling must be normalized to ensure rhythmic circadian activity in adulthood. The pupal stage is a period of massive neuronal reorganization and since insulin signaling also has an important role in neural development (FERNANDEZ AND TORRES-ALEMAN 2012), aberrant insulin signaling could result in improper reorganization of the circadian output circuit during metamorphosis. Interestingly, the pupal stage is also a time period during which neurons involved in the circadian molecular clock circuit migrate and divide into their eventual adult locations and numbers (EGGER *et al.* 2008). We know that defects in the clock output circuit are responsible for circadian arrhythmicity in *dfmr1* mutant flies (DOCKENDORFF *et al.* 2002), although the neurons involved in this circuit have not yet been fully defined. It is likely that dysregulation of insulin signaling during the pupal stage would affect the proper development of the neurons in this circadian output pathway, setting the stage for arrhythmic circadian behavior in adulthood.

Interestingly, memory defects in *dfmr1* mutants do not appear to have as strong a developmental component. In Chapter 4 we showed that normalizing insulin signaling in adulthood is sufficient to restore both courtship-based and olfactory-based memory. Furthermore, we showed that metformin treatment acutely in adulthood could rescue both forms of memory. Our results are concordant with previous studies suggesting that memory can be rescued during adulthood in *dfmr1* mutant flies and *Fmr1* knockout mice (MCBRIDE *et al.* 2005; BOLDUC *et al.* 2008; GUO *et al.* 2011; MICHALON *et al.* 2012). Our results suggest that the defects underlying memory deficits in *dfmr1* mutants are not grounded in circuit-level problems caused by signaling imbalances during development, but rather are rooted in current signaling imbalances. Thus these defects are more amenable to treatment because a relatively simply rebalancing of signaling will ameliorate them.

However, in must be noted that we also observed that metformin treatment during development alone rescued STM in the conditioned courtship assay. This result indicates that STM defects have both a developmental and physiological component. Altogether, these results

suggest a model in which misregulation of insulin signaling results in formation of neural circuitry defects during development, which leads to defects in circadian behavior and STM. In contrast, misregulated insulin signaling directly contributes to olfactory-based memory defects. While the circuitry defects underlying circadian arrhythmicity are sufficient to cause arrhythmic behavior, the STM circuitry defects are not, but together with continual misregulation of insulin signaling, they result in deficits in STM. Thus rescue of either circuitry defects during development, or signaling defects during adulthood, can ameliorate STM in *dfmr1* mutant flies. Conversely, since circadian arrhythmicity and olfactory-based memory defects result from only circuitry or physiological signaling defects respectively, only developmental or adulthood treatment will rescue these defects. It has previously been shown that acute insulin administration can affect memory (KIM AND FELDMAN 2015), so it follows both that misregulated insulin signaling could depress memory, and that acute rebalancing of insulin signaling could restore memory.

Interestingly, we discovered that while courtship-based memory could be rescued by metformin treatment in both development and adulthood, olfactory-based memory could only be rescued by metformin treatment in adulthood. The divergence in the timing of rescue of these two forms of memory may be due to differences in the inputs required for formation of these memories. Olfactory-based memory formation is solely dependent on an olfactory stimulus, while courtship-based memory involves a combination of olfactory, gustatory, visual and social inputs (DAVIS 2005; GRIFFITH AND EJIMA 2009). Since not all these inputs need to be present for courtship-based memory formation (JOINER ML AND GRIFFITH 1997), we propose that some of these inputs may be rescued by developmental metformin treatment, while others may be rescued by physiological metformin treatment, thus allowing rescue of courtship-based memory to occur at several different time points.

These results confirm the strong suspicion that some FXS traits may be established in development due to improper neural wiring, and we may need to search for the downstream repercussions of these wiring defects to treat them. Here we have shown that merely targeting insulin signaling may not be enough to rescue behavioral problems in *dfmr1* mutant flies. It is

critical to establish the essential timing of treatment. Furthermore, understanding the essential developmental timing of the treatment will make it easier for us to identify a mechanism, which may ultimately allow us to develop better treatments.

Implications of metformin treatment for FXS:

In Chapters 3 and 4 we identified that metformin treatment was capable of rescuing memory, but not circadian behavior. Our discovery of the drug metformin as an enhancer of two forms of memory in *dfmr1* mutant flies may provide some clues as to the mechanism of insulin signaling action on memory. The identification of metformin is particularly exciting because as a FDA approved drug, it could be used immediately in patients as long as metformin treatment is also effective in rescuing behavioral deficits in *Fmr1* KO mice.

However, although metformin is known to affect the insulin signaling pathway, its mechanism of action is currently unknown. Previous studies have shown that metformin raises pAMPK levels, which has the effect of reducing insulin signaling downstream of the TOR pathway (SLACK *et al.* 2012). It should be noted, however, that metformin also acts through other pathways, including through cAMP and mitochondrial complex I (MILLER *et al.* 2013; HUR AND LEE 2015). Metformin is able to cross the blood-brain barrier, indicating that it could act in the nervous system to produce its effects (Heni et al. 2015). Interestingly, the metformin developmental treatment profile coincides with the developmental profile of insulin signaling normalization in that adulthood treatment is sufficient to rescue memory in both cases. These results argue that metformin most likely acts through a mechanism that more directly normalizes insulin signaling, rather than a more obscure mechanism that works indirectly. Currently we can only speculate as to which pathways metformin targets to improve memory in *dfmr1* mutant flies, but further pursuit of this question will be important to understanding the mechanism by which memory is disrupted in FXS.

It should be noted that we were unable to rescue circadian behavior with metformin treatment given during either development or adulthood. While the first explanation for this finding

may be that metformin treatment simply does not target the underlying deficits causing circadian abnormalities, there is another explanation. Remarkably, this finding may be explained by the discovery that rescue of insulin signaling during the pupal period is required for normal circadian behavior. If metformin acts by normalizing insulin signaling, and insulin signaling must be normalized during the pupal stage to rescue circadian behavior, it follows that metformin would also likely need to be administered during the pupal stage to rescue circadian behavior. Since we deliver drug treatments through food, we are unable to continue treatment during metamorphosis when flies cease eating to undergo pupation, suggesting that our inability to deliver drug treatment during the pupal stage, rather than the ineffectiveness of the drug, may be the primary reason for the inability of metformin to rescue circadian behavior.

Curiously, there have been previous instances where drug treatment rescues memory but not circadian behavior. Specifically, mGluR inhibitors rescue memory in *dfmr1* mutants, but do not rescue circadian rhythmicity (McBride *et al.* 2005; Bolduc *et al.* 2008). It seems quite possible that if the major defect underlying circadian arrhythmicity results from misregulated signaling during the pupal period, that any treatment attempting to restore rhythmicity would have to be administered during this critical period. These observations strongly hint that examining insulin signaling during the pupal period may be the key to understanding circadian arrhythmicity in *dfmr1* mutant flies.

Future Directions:

Our findings uncover a new pathway affected by loss of *dfmr1* that is important for maintaining normal circadian behavior and memory in *Drosophila.* However, they leave us with a number of questions that need to be addressed.

First, we need to determine the mechanism by which dFMR1 acts in the IPCs to promote normal insulin signaling and behavior. Whether dFMR1 in the IPCs represses translation of *dilp2,* regulates neural morphology, represses mGluR signals that promote insulin release, or acts through the miRNA pathway to repress genes involved in insulin signaling, determining the

mechanism of action will give us crucial information about what to look for when we examined FMR1 function in the β-cells and neurons of mammals.

Second, we need to develop a multi-dimensioned map of the insulin signaling profile of *dfmr1* mutant flies. It is important to understand that timing, level, and location of aberrant insulin signaling in *dfmr1* mutant flies if we are to fully understand the origins and most effectively target the problem. This information will tell us when and where to direct treatment, and will help us judge the magnitude of intervention required to successfully address the problem.

Third, we need to develop a better understanding of the precise mechanism by which aberrant insulin signaling leads to memory and circadian defects in *dfmr1* mutants. Understanding these mechanisms will help us to pinpoint places possibly amenable to treatment, and to identify new pathways to target for treatment.

Fourth, we need to examine insulin signaling in patients with FXS and in *Fmr1* knockout mice. Our findings leave us with the tantalizing suggestion that insulin signaling may be disrupted in mammals when *Fmr1* is absent, but further experiments need to be done to confirm this possibility. These experiments will aid us in determining the degree to which insulin signaling defects identified in *dfmr1* mutants translate to mammals.

Finally, we need to determine the mechanism by which metformin rescues memory in *dmr1* mutants. If metformin rescues memory in an indirect fashion it will inform of another pathway affected in FXS, and may provide us with a new series of treatment opportunities. Regardless, metformin could be a valuable therapy for FXS patients, and should be tested in *Fmr1* knockout mice to determine if its efficacy is conserved.

Conclusions:

Here we show that normal insulin signaling is disrupted in *dfmr1* mutant flies, and that normalization of insulin signaling through genetic or pharmacological means rescues circadian behavior and memory. Clearly further investigation will help unravel the connection between insulin signaling, memory and circadian behavior, and establish how metformin affects memory in

dfmr1 mutants. Exploration of these results in the mouse fragile X model and fragile X patients will be essential, as such investigations may lead to novel treatments for FXS. Importantly, FXS patients are not the only ones that will benefit from understanding the signaling pathways underlying behavioral difficulties caused by loss of *Fmr1.* As a major cause of intellectual disability and autism, FXS has the potential to give us insight into the mechanisms at the root of numerous other diseases with overlapping symptoms. We should therefore remember that the development of new treatments for FXS would have consequences for more than the already abundant 1 in 5000 individuals and their families affected by FXS worldwide.

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Appendix 1: Further investigation of insulin signaling in *dfmr1* mutants

Abstract:

In the previous chapters we determined that insulin signaling is increased in *dfmr1* mutants, and that ameliorating this increase rescues memory and circadian behavior. However, we have seen indications that insulin signaling in *dfmr1* mutants may be more complicated than previously thought.

We found that induction of insulin resistance using a high sugar diet rescued circadian behavior in *dfmr1* mutants, suggesting that any form of insulin signaling reduction may be able to rescue circadian rhythmicity. We also found that decreasing insulin signaling in *dfmr1* mutants exacerbated the mushroom body (MB) crossover phenotype, indicating that reduction of insulin signaling is not capable of rescuing all *dfmr1* mutant phenotypes.

Finally, we examined brains stained with an anti-Dilp2 antibody and observed a large number of puncta that stained positive for Dilp2. We found that these puncta appeared to behave differently in different regions of the brain. In the subesophageal region of the brain, puncta levels were decreased in *dfmr1* mutants, while in the supraesophageal region, the puncta levels did not change in the absence of *dfmr1*. Interestingly, when we expressed *dfmr1* in the IPCs of otherwise *dfmr1* mutant flies, puncta levels decreased in both regions of the brain. However, the most robust decrease occurred in the subesophageal region.

From these experiments it is clear that the regulation of insulin signaling is intricate, and it may take close temporal and spatial examination of *dfmr1* mutants to truly understand how loss of *dfmr1* affects behavior.

Introduction:

In the previous chapters, we presented evidenced that insulin signaling is disrupted in a *Drosophila* model of Fragile X syndrome (FXS), and that this disruption in signaling contributes to circadian and memory abnormalities. We showed that *dfmr1* expression in the insulin-producing cells (IPCs) was important for both courtship-based and olfactory-based memory, as well as for circadian behavior. Furthermore, we found evidence of increased *Drosophila* insulin-like peptide (Dilp2) levels in the IPCs, and increased phosphoinositide 3-kinase (PI3K) and Akt activity in other regions of the brain, indicating that loss of *dfmr1* results in increased insulin signaling. Normalizing insulin signaling by genetic reduction rescued circadian behavior and both forms of memory tested, confirming that misregulated insulin signaling contributes to the behavioral phenotypes of the *dfmr1* mutant fly.

Our results that insulin signaling is disrupted in the fly model of FXS suggest that abnormal insulin signaling may be a pathogenic factor in the disease. Investigation of literature on FXS patients reveals several hints that metabolism is abnormal following loss of *FMR1* function. Slightly less than 10% of patients with FXS display a distinctive Prader-Willi phenotype in which insatiable appetite and obesity are hallmarks (McLennan *et al.* 2011). Patients also seem to be more susceptible to the uncontrollable weight gain associated with anti-psychotics, suggesting that they may have a genetic propensity for obesity (McLennan *et al.* 2011). Perhaps counterintuitively, males with FXS exhibit significantly lowered total cholesterol levels, which are independent of their BMI (Berry-Kravis *et al.* 2015). Clearly there is evidence of metabolic dysfunction in FXS, but the rather sparse data do not suggest a generally increase insulin signaling. Rather, they suggest that metabolic dysregulation in FXS is more complicated.

Given this evidence, it is clear we need to expand our understanding of insulin signaling in *dfmr1* mutant flies. Indeed, although we showed noted increased insulin signaling in the mutants, they do not appear larger than other flies, as would be expected if insulin levels were ubiquitously increased (Brogiolo *et al.* 2001; Ikeya *et al.* 2002). These results suggest that further assessment of insulin signaling in *dfmr1* mutants will be informative.

In this appendix, we show that dietary induction of insulin resistance, which results in an effectual decrease in insulin signaling, rescues circadian behavior in *dfmr1* mutants. This result suggests that multiple mechanisms can be used to alter the insulin signaling pathway and achieve changes in behavior. We also show that reduction of insulin signaling exacerbates the mushroom body (MB) crossover phenotype of *dfmr1* mutants, implying that phenotypic deficits of this fly model are not entirely due to increased insulin signaling, and that insulin signaling misregulation in these flies may encompass both increases and decreases in the pathway. Finally, we show that Dilp2 puncta quantity and distribution are altered by loss of *dfmr1* and by expression of *dfmr1* in the IPCs, suggesting that *dfmr1* has a role in determining not just the amount, but also the location of insulin release, and thus proposing a novel and more complicated mechanism by which loss of *dfmr1* could disrupt insulin signaling. Altogether these data indicate that the insulin signaling environment of *dfmr1* mutants is complex and warrants increased examination if we want to fully understand its contribution to FXS.

Results:

A high sucrose diet rescues circadian behavior in dfmr1 mutants

In Chapter 3 we showed that misregulated insulin signaling in *dfmr1* mutants contributes to the abnormal circadian behavior and memory seen in this fly model of FXS. We found that reducing insulin signaling both ubiquitously and pan-neuronally in *dfmr1* mutant flies was sufficient to restore normal circadian rhythmicity and memory in both the conditioned-courtship paradigm and the olfactory conditioning paradigm. Furthermore, normalizing insulin signaling with the type 2 diabetes drug metformin was able to rescue both forms of memory, but did not rescue circadian behavior. We then wondered if we could rescue circadian behavior by reducing insulin signaling through dietary induction of insulin resistance. It has previously been shown that feeding flies a high sucrose diet results in insulin resistance as measured by decreased body size and weight, an increase in both circulating sugar and insulin, and a failure of the fat body to respond to insulin stimulation (PASCO AND LEOPOLD 2012). To test whether a high sugar diet could affect

circadian rhythmicity in *dfmr1* mutants, we raised *dfmr1* mutants and wild-type controls on fly food containing five-times the sucrose of normal food (5X sucrose) and examined circadian behavior. We found that a 5X sucrose diet restored circadian rhythmicity to normal in *dfmr1* mutants while having little effect on wild-type controls (Figure A-1). These results confirm our previous findings that reducing insulin signaling rescues circadian behavior in *dfmr1* mutants.

Reduction of insulin signaling exacerbates mushroom body crossover in dfmr1 mutants

Our previous findings that genetic reduction of insulin signaling rescued circadian and memory deficits in *dfmr1* mutants led us to examine whether the same genetic manipulations would rescue a morphological defect seen in *dfmr1* mutant flies. *Dfmr1* mutants have been reported to exhibit increased incidence of crossover of the beta lobes of the mushroom body (MB) (MICHEL *et al.* 2004). We score this crossover as being anywhere from absent, in which the MBs appear normal, to severe, in which the beta lobes are completely fused together (Figure A-2). To determine if reduction of insulin signaling reduces the propensity of *dfmr1* mutants to exhibit MB crossover, we examined α-Fasciclin II stained brains from *dfmr1* mutants with reduced levels of *dilp2, dilp3, dilp5,* or *dilp2&3.* Surprisingly, not only did reduction of *dilp* levels fail to reduce the incidence of crossover, reduction in *dilp3* actually intensified the phenotype (Figure A-3). To confirm this finding, we examined *dfmr1* mutant brains with either pan-neuronally reduced PI3K activity, or pan-neuronal overexpression of PTEN. Again, a decrease in insulin signaling appeared to be at best neutral and at worst to have a detrimental effect on MB crossover (Figure A-4). Interestingly, expression of $DP110^{DN}$, which appears to have a more depressive effect on insulin signaling than PTEN overexpression, resulted in a stronger aggravation of the crossover phenotype than overexpression of PTEN, implying that decreasing insulin signaling worsens MB cross-over in a dose-dependent manner. These findings also raise the possibility that the insulin signaling misregulation seen in *dfmr1* mutants may dissimilarly affect different areas of the brain.

Dfmr1 affects quantity of Dilp2 puncta in brain

To form a more defined picture of Dilp2 signaling in *dfmr1* mutants, we stained brains with a Dilp2 antibody and examined Dilp2 distribution. We were intrigued to discover a large number of puncta on either side of the IPCs and in the subesophogeal region of the brain that were specifically stained for Dilp2 (Figure A-5a, Figure A-6a). Quantification of the puncta in the subesophogeal region revealed that significantly fewer puncta were present in *dfmr1* mutants than in wild-type controls (Figure A-5a & b). We then quantified the puncta located on either side of the IPCs, positioned slightly dorsally between the *pars intercerebralis* and the optic lobes. We were unable to detect any difference in the number of these puncta between the *dfmr1* mutants and wild-type controls (Figure A-6a & b). These results suggest that loss of *dfmr1* alters levels of Dilp2 in the brain in a manner that affects distribution to specific regions of the brain differently.

We then queried how expression of *dfmr1* in the IPCs would alter Dilp2 puncta. When we quantified Dilp2 puncta in the subesophogeal region of brains which expressed *dfmr1* in the IPCs, we observed that surprisingly, puncta number was significantly decreased compared to *dfmr1* mutant controls (Figure A-7a & b). Similarly, expression of *dfmr1* in the IPCs also appears to result in a decrease in Dilp2 puncta in the region dorsal of the subesophogeal region (Figure A-8a & b), though comparison to the *dfmr1* mutant control with the *UAS-dfmr1* transgene alone did not reach significance, potentially because of leaky expression of the UAS. These results suggest that *dfmr1* expression in the IPCs suppresses the number of Dilp2 puncta in all regions of the brain. The finding that expression of *dfmr1* in the IPCs reduces Dilp2 puncta in the subesophogeal region even farther below the reduction caused by loss of *dfmr1* is curious. It seems counterintuitive that a phenotype caused by loss of *dfmr1* would be worsened by re-expression of *dfmr1*, but it is important to be cognizant that the loss of *dfmr1* affected all cells, whereas the gain of *dfmr1* occurred only in the IPCs, thus the result that expression of *dfmr1* in the IPCs further decreases the number of Dilp2 puncta in the subesophogeal region suggests *dfmr1* expression in other cells in the brain is important for regulating quantity of Dilp2 puncta.

Discussion:

In this appendix, we showed that dietary-induced insulin resistance rescues circadian behavior in *dfmr1* mutants, confirming our previous finding that reducing insulin signaling ameliorated circadian rhythmicity. Conversely, we determined that reducing insulin signaling in *dfmr1* mutants actually had a deleterious effect on MB crossover, suggesting that the previously observed increased insulin signaling in *dfmr1* mutants may not be pervasive, and that the insulin signaling environment of mutant flies could be heterogeneous. Finally, we identified a role for *dfmr1* in regulating distribution of Dilp2 puncta to different regions of the brain, and in repressing Dilp2 puncta levels when expressed in the IPCs. These results imply that *dfmr1* mutants experience variable levels of insulin signaling in different brain regions and that understanding how dFMR1 regulates will be essential to understanding the role of insulin signaling in FXS.

In this appendix, we describe the interesting finding that inducing dietary insulin resistance in *dfmr1* mutants rescues circadian behavior deficits. Our experiment is not the first time diet has been shown to affect phenotypes in *dfmr1* mutants (CHANG *et al.* 2008), but it is the first time that diet which alters the insulin signaling pathway has been shown to rescue mutant phenotypes. Obviously inducing insulin resistance is not a practical therapy for FXS, but our finding does confirm our previous observations that reduction of insulin signaling improves circadian behavior in *dfmr1* flies. Since a 5X sucrose diet induces insulin resistance before pupation (PASCO AND LEOPOLD 2012), this result is also consistent with our previous finding that insulin signaling must be reduced during the pupal period to rescue circadian behavior. It should be noted that we have not yet established that *dfmr1* mutants develop insulin resistance on the 5X sucrose diet by confirming that tissues cease to respond to insulin, however we did observe a noted decrease in size of pupae on the 5X food which suggests that insulin resistance occurs in *dfmr1* mutants. In sum, this result corroborates our previous findings, and suggests that properly regulating insulin signaling in some areas may have unanticipated negative effects on other areas.

A perfect example of how reduction in insulin signaling can have both positive and negative effects on *dfmr1* mutants is our finding that the MB morphology phenotype is exacerbated by reduced insulin signaling. One possible explanation for this seeming anomaly is that increased insulin signaling does not affect every part of the brain in *dfmr1* mutants, thus it may not be a causative factor in the MB crossover phenotype. Another possibility is that the MBs are more sensitive to increased insulin signaling, possibly resulting in insulin resistance in this particular group of neurons. If lowered insulin signaling due to insulin resistance was a causative factor for MB crossover, it would be reasonable that reducing insulin signaling could exacerbate crossover as we observed. However, arguing against this possibility is our finding in Chapter 2 that PI3K and Akt activity were increased in the MB calyx region, which would indicate that these MB cells are not insulin resistant in adulthood. Nevertheless, we did not examine insulin signaling in all cells of the MB, so the potential remains that insulin resistance could exist in a subset of cells that were not imaged.

These speculations suggest a series of experiments to help us better understand the contribution of insulin signaling to MB morphology. If the MB crossover phenotype is actually the result of regional low insulin signaling in *dfmr1* mutants, we would expect that increasing insulin signaling either pan-neuronally or in the MBs specifically might rescue the crossover phenotype. Similarly, if reduced insulin signaling is the origin of MB crossover, we would expect that reducing insulin signaling either pan-neuronally or specifically in the MBs might increase the incidence of MB crossover in wild-type flies. Another question is whether worsened MB crossover stimulated by reduced insulin signaling is the result of reduced signaling throughout the brain, or reduced insulin signaling specifically in the MB. To address this question we can reduce insulin signaling specifically in the MB of *dfmr1* mutants and examine whether aggravation of crossover still occurs.

Another interesting observation from the MB data is that reduction of different Dilps did not have the same effect. Specifically, while reduction of Dilp2, Dilp5, or Dilp2-3 resulted in no effect on MB crossover, reduction of Dilp3 resulted in over 50% of MBs displaying severe

crossover, suggesting that Dilp3 in particular might be an important component of the interaction between the insulin signaling pathway and MB crossover. Henceforth our studies have largely focused on Dilp2, but it is just one of several Dilps synthesized in the IPCs (BROGIOLO *et al.* 2001). Clearly to achieve a more comprehensive understanding of insulin signaling in *dfmr1* mutants, we will need to examine how Dilp3 and Dilp5, and ultimately all 8 Dilps, play a role in neural regulation in and around the brain.

A final apparent discrepancy in our finding that reduced insulin signaling does not rescue MB morphology is that expression of *dfmr1* in the IPCs does rescue MB morphology (unpublished data). In previous chapters we showed that expression of *dfmr1* in the IPCs lowered insulin signaling in the brain, the MB findings suggest that *dfmr1* may function in the IPCs to modulate insulin signaling in different areas of the brain.

Our observations of the effect of *dfmr1* on Dilp2 puncta quantity and distribution support the idea that dFMR1 functions in and outside of the IPCs to balance insulin signaling in the brain. In this appendix we observed that in *dfmr1* mutant brains, Dilp2 puncta were reduced in the subesophogeal region, but unaffected in the supraesophogeal region. However, when *dfmr1* was expressed in the IPCs, both brains regions showed a decrease in Dilp2 puncta. These findings suggest a complex role for *dfmr1* in modulation of Dilp2 levels and distribution in the brain.

A key question raised by our findings is precisely what the Dilp2 puncta represent. They do not appear to be in projections of the IPCs we normally visualize when staining, but it seems unlikely that they represent freely moving clusters of Dilp2 existing outside the IPCs. Considering the orderly lines formed by the puncta, especially in the region above the esophagus, it seems probable that the puncta are moving through neural projections that serve as paths for small, regulated clusters of Dilp2, and that therefore do not appear as bright, continuous lines as the projections around the IPC cell bodies do. To better understand the role these Dilp2 punta play in insulin signaling, we can depolarize the IPCs using the bacterial sodium channel (NaChBac) to determine whether activating the IPCs results in disappearance, and thus probable release of some puncta. Conversely, we can hyperpolarize the IPCs by expressing the constitutively active

potassium channel, Kir2.1, and determine if the Dilp2 puncta increase in number. It would also be informative to determine how the puncta respond to nutrient signals. Starvation causes Dilp2 accumulate in the cell bodies of the IPCs, and feeding reverses this accumulation (GEMINARD *et al.* 2009), thus it would be interesting to determine if Dilp2 puncta increase in response to starvation and decrease in response to refeeding. These experiments will aid us in determining if the presence of these puncta represents an increase in insulin signaling, and is a sign that Dilp2 is being actively transported to release points, or if they represent a decrease in insulin signaling, such that the Dilp2 are being retained in the cell and not released. Clearly further experimentation will be important to establish the precise function of the Dilp2 puncta in overall insulin signaling from the IPCs.

Our experiments indicate that in *dfmr1* mutants, Dilp2 puncta are significantly decreased in the subesophogeal region. If we determined that the decreased level of puncta signifies a release of Dilp2 into the region, and thus an increase in insulin signaling in that region, this finding could be consistent with our previous findings that insulin signaling is increased in the brains of *dfmr1* mutants. Conversely, if we determine that a decreased number of puncta in this region is consistent with decreased transport of Dilp2 to the region and decreased release of Dilp2, our finding implies that insulin signaling may be decreased in the subesophogeal region of *dfmr1* mutant brains, indicating that different regions of the brain may experience dissimilar insulin signaling levels. Interestingly, the subesophogeal region is known to contain neurons important for feeding behavior and courtship (MANN *et al.* 2013; TRAN *et al.* 2014; HUCKESFELD *et al.* 2015), leading to the intriguing possibility that the IPCs may signal to these neurons to regulate feeding and courtship behavior.

Our results also indicate that expression of *dfmr1* in the IPCs results in reduced Dilp2 puncta throughout the brain. However, in the subesophogeal region this finding represents an exacerbation of the puncta phenotype we observed in *dfmr1* mutants, raising the question of how to reconcile these apparently divergent findings. The resolution to this question may require understanding how dFMR1 functions in the IPCs to alter puncta number. dFMR1 is known to be

important for efficient transport of RNA along microtubules in association with dynein and kinesin motor proteins (ESTES *et al.* 2008), raising the possibility that dFMR1 assists in transport of *dilp2* mRNAs to a location where they are locally transported. Interestingly, excessive levels of dFMR1 also interfere with transport, possibly due to an increased propensity for dFMR1 to homodimerize instead of continuing its function in transport (ESTES *et al.* 2008). This tendency could explain our conflicting subesophogeal staining results, because a loss of *dfmr1* and an overexpression of *dfmr1,* which may occur in the IPCs with the *dilp2-Gal4* driver, would be expected to result in decreased transport of mRNAs. We could test whether kinesin or dynein were important for localization of Dilp2 puncta by knocking down either of the proteins in the IPCs and examining Dilp2 puncta localization.

Another possible explanation for the anomalous finding that expression of *dfmr1* in the IPCs further reduces Dilp2 puncta number in the subesophogeal region, is that Dilp2 puncta number in this region of the brain is also regulated in the opposite direction by expression of *dfmr1* in other neurons in the subesophogeal region. For instance, it appears that expression of *dfmr1* in the IPCs instigates reduction of Dilp2 puncta in the area surrounding the IPCs. However, dFMR1 expression may be required in neurons in the subesophogeal region to promote Dilp2 puncta formation or movement to the subesophogeal region, resulting in a tension between dFMR1 in the IPCs and dFMR1 in other neurons. Because dFMR1 has many RNA targets, it might directly suppress Dilp2 translation in the IPCs while indirectly promoting Dilp2 accumulation by binding to other RNA targets in other subesophogeal neurons. If this hypothesis that *dfmr1* expression is required in both the IPCs and subesophogeal neurons to promote wild-type Dilp2 puncta quantity in the subesophogeal region, then expression of *dfmr1* pan-neuronally should restore wild-type levels of Dilp2 puncta to the subesophogeal region. Clearly the results of the Dilp2 staining raise interesting questions about how dFMR1 affects insulin signaling in the brain, and it will be imperative that we determine the answers to these questions so we can move beyond speculation and develop a better understanding the interconnecting interactions of insulin signaling and *dfmr1*.

One final interesting implication of the Dilp2 puncta is the possibility that Dilp2 is being released locally in the brain from the IPCs. If Dilp2 is only released onto the corpora cardiac and into the hemolymph, it would seem illogical that Dilp2 would be transported throughout the brain if it only needed to be transported in one direction. Local Dilp release in the brains of *Drosophila* is not entirely unprecedented. Although most insulin signaling in the fly occurs when the IPCs release insulin into the hemolymph to be carried throughout the body (NASSEL *et al.* 2013), a small subset of insulin is released directly into the brain itself. Dilp2 is released into cells in the brain that express Imp-L2, a Dilp2 binding protein, during the larval period (BADER *et al.* 2013). These cells must be near the IPCs for Dilp2 to be absorbed by Imp-L2-expressing cells (BADER *et al.* 2013), suggesting that the IPCs release Dilp2 locally as well as systemically. Interestingly, the IPCs are not the sole insulin secreting cells in the brain. Two studies identified a group of glial cells that release Dilp6 and Dilp2 to stimulate neuroblast reactivation in larvae (CHELL AND BRAND 2010; SOUSA-NUNES *et al.* 2011). These Dilps originate from a completely separate insulin pool than those released by the IPCs (SOUSA-NUNES *et al.* 2011), indicating that the IPCs are not involved in release of these Dilps. If local release of Dilp is dysregulated in *dfmr1* mutants, it would not only make it extremely hard to recapitulate the spatial profile of insulin signaling in these flies, but it would also suggest that treatment for defects caused by excessive insulin signaling would be more complicated than just reducing insulin signaling. If only a portion of the brain was subject to increased insulin signaling, reducing insulin signaling throughout the brain might cause unforeseen side effects.

However, while the idea of local insulin release is intriguing to consider in *Drosophila*, there are no accounts of insulin release occurring in the adult brains of mammals. Mammals do produce insulin and IGF1 and IGF2 in their brains during development (FERNANDEZ AND TORRES-ALEMAN 2012; BLAZQUEZ *et al.* 2014; GRAY *et al.* 2014). However, while both IGF-1 and IGF-2 are produced in the adult mammalian brain, albeit at lower levels than during development, insulin production in the adult brain has not been definitely demonstrated (FERNANDEZ AND TORRES-ALEMAN 2012; HENI *et al.* 2015). Nonetheless, there are hints that insulin mRNAs are produced in

the mammalian brain even in adulthood (BLAZQUEZ *et al.* 2014; GRAY *et al.* 2014), suggesting that we may still have things to learn about insulin signaling in the brain. Indeed, it was only recently discovered that *Drosophila* display local release of insulin into their brains (CHELL AND BRAND 2010; SOUSA-NUNES *et al.* 2011; BADER *et al.* 2013), raising the possibility that insulin is synthesized at low levels in the brains of mammals but has not yet been discovered. Any insulin made in the brain is probably made at extremely low levels. Only a fraction of stored insulin is released into the blood from the β-cells, yet this small amount of insulin is sufficient to aid glucose uptake in an entire organism (Rutter et al. 2015). Thus the amount of insulin necessary to signal to a small number of cells would be miniscule and extremely difficult to detect. It is also interesting to consider that since insulin-producing cells have their evolutionary origins in the brain (OTTER AND LAMMERT 2016), perhaps some remained there even when the major location of insulin production was shifted to the pancreas. The idea of insulin production in the brain is certainly intriguing to speculate about, and misregulation of insulin signaling in the brain could have interesting implications for FXS patients.

In this chapter, we showed that dietary insulin resistance could improve circadian behavior in *dfmr1* mutants, confirming previous results that decreased insulin signaling restores circadian behavior. We also showed that reducing insulin signaling in *dfmr1* mutants fails to rescue MB morphology, indicating that insulin signaling in *dfmr1* mutants may be differentially misregulated in different areas of the brain. Finally, we show that dFMR1 regulates levels of Dilp2 puncta to different degrees depending on the brain region in which the puncta are located. Altogether, these results indicate that *dfmr1*mutants may experience a much more complicated insulin signaling environment in their brains than previously thought, and that through a better understanding of the magnitude and spatial parameters of this insulin signaling, we will be better able to specifically reverse misregulated insulin signaling to improve behavior and morphology in the brains of the FXS fly model. These results indicate working to better understand the complex insulin signaling environment of *dfmr1* mutant flies will be important to developing more targeted treatments that take into account the heterogeneity in misregulation of signaling pathways in FXS.

Figures:

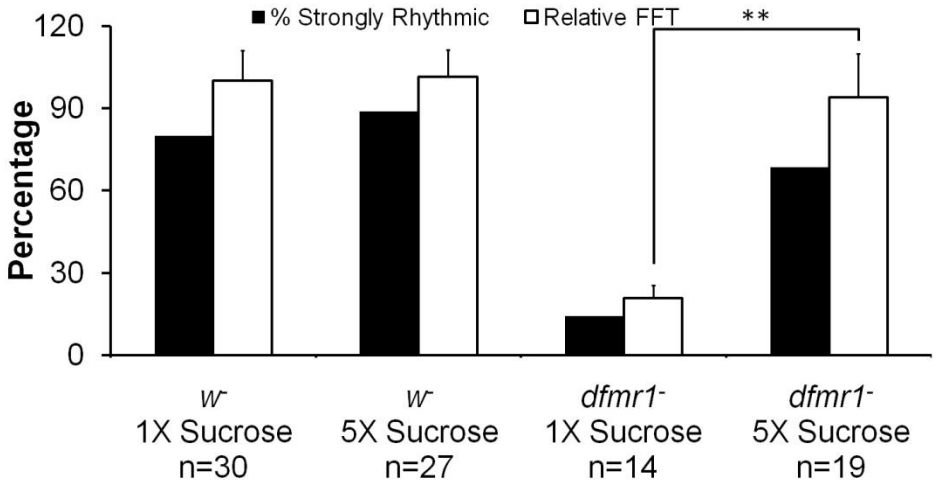

Figure A-1. *Dfmr1* mutants raised on a high sucrose diet show significantly improved circadian rhythmicity. Graph depicts percentage of strongly rhythmic flies (FFT > .04) (black) and relative FFT (white). Relative FFT represents how the average FFT of the depicted genotype compares to the average FFT of a wild-type control. *Dfmr1* mutants and wild-type controls were raised on either 1X or 5X sucrose food, then their circadian rhythmicity was assessed. Percentage of strongly rhythmic flies and relative FFT were improved in *dfmr1* mutants raised on 5X sucrose food. Rhythmicity was determined by FFT and visual inspection of the actogram and periodogram. Statistical significance is denoted with asterisks (*p<0.05, ** p<0.01, ***p<0.001). Statistical significance was determined by a Kruskal-Wallis test and Dunn's post-test. Graphs depict mean ± s.e.m. These data were collected in collaboration with Brian DeBaun.

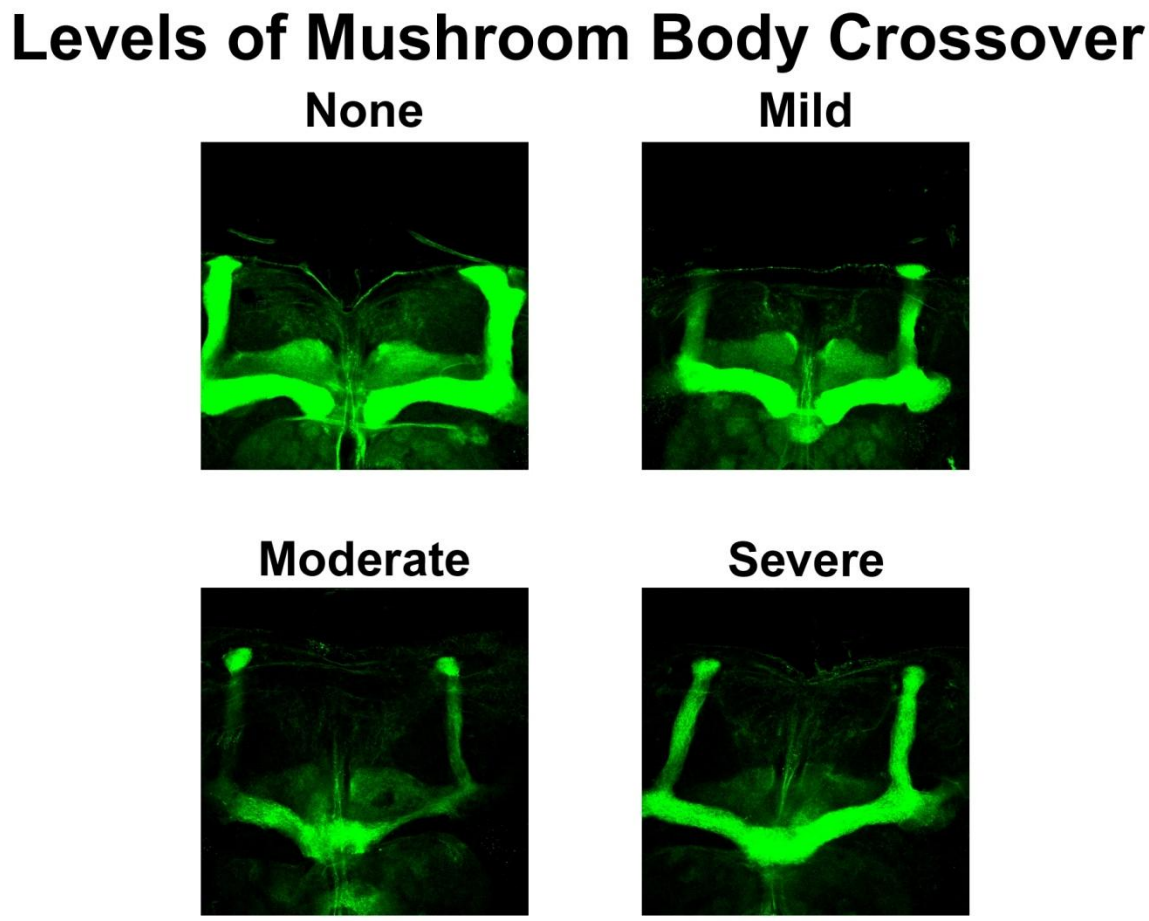

Figure A-2. Various degrees of mushroom body crossover. Mushroom body crossover occurs when the normally separated lobes of the mushroom bodies exhibit varying degrees of fusion. Mild crossover occurs when a small segment of the beta lobes is fused. Moderate crossover occurs when there is substantial, but not complete fusion of the mushroom bodies. Severe crossover occurs when the beta lobes are completely fused.

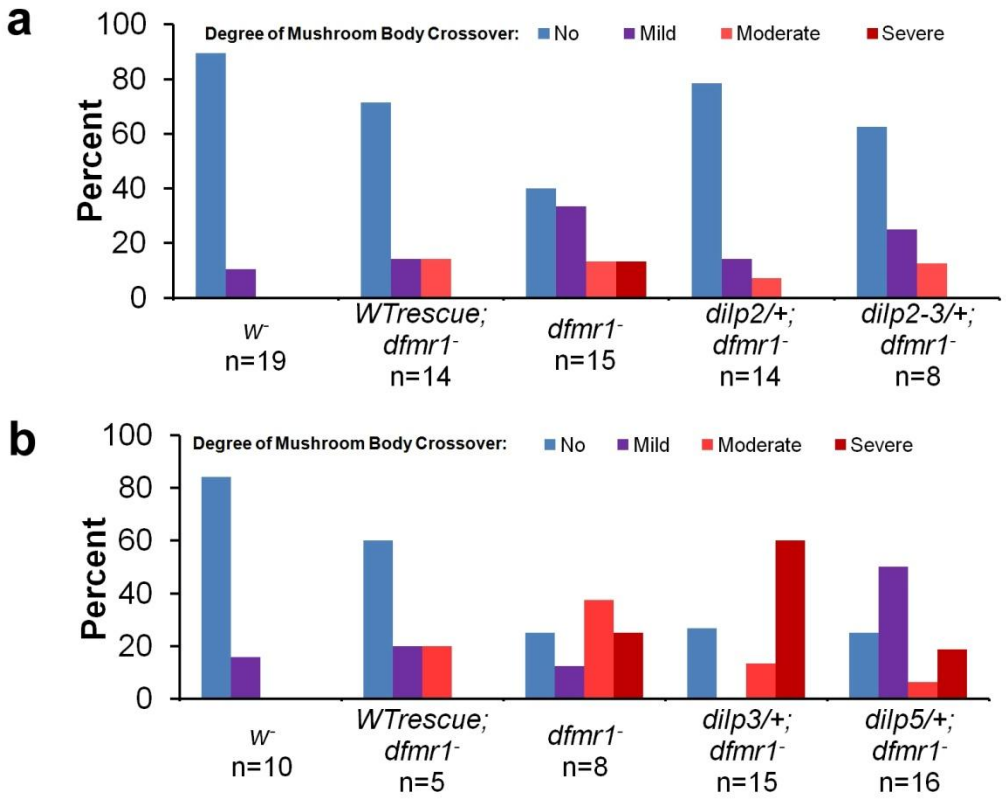

Figure A-3. Reduction of *dilp* expression in *dfmr1* mutants does not change or worsens the mushroom body crossover phenotype. Brains were dissected and stained with α-Fasciclin II to illuminate the mushroom body morphology. Brains were imaged and scored for mushroom body crossover blind to genotype. **(a)** *Dfmr1* mutants heterozygous for *dilp2* or *dilp2-3* did not show substantial improvement in mushroom body crossover. **(b)** *Dfmr1* mutants heterozygous for *dilp3* or *dilp5* exhibit no improvement or worsening of mushroom body crossover.

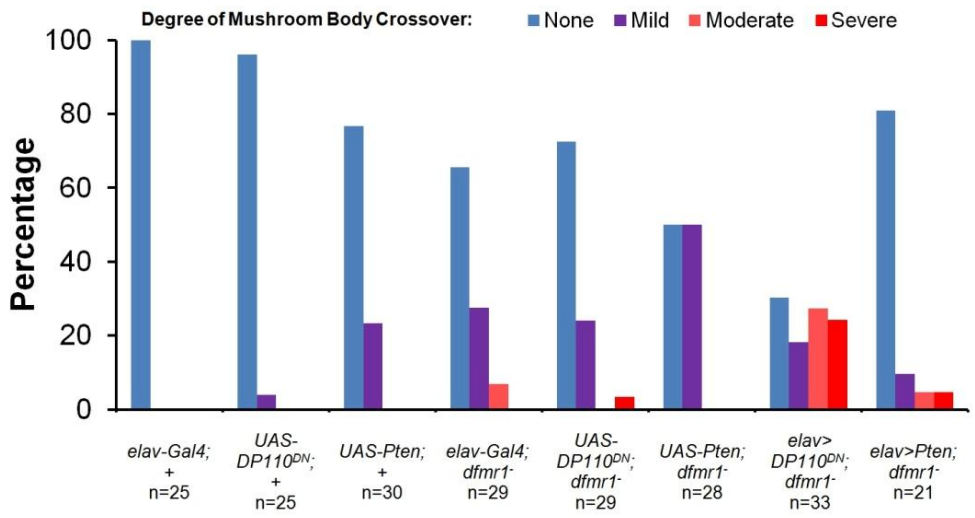

Figure A-4. Pan-neuronal reduction of insulin signaling in *dfmr1* mutants fails to rescue or aggravates mushroom body crossover. Brains were dissected and stained with α-Fasciclin II to illuminate the mushroom body morphology. Brains were imaged and scored for mushroom body morphology blind to genotype. Pan-neuronal expression of $DP110^{DN}$ in *dfmr1* mutants exacerbated the mushroom body crossover phenotype, and pan-neuronal expression of PTEN in *dfmr1* mutants did not improve mushroom body crossover.

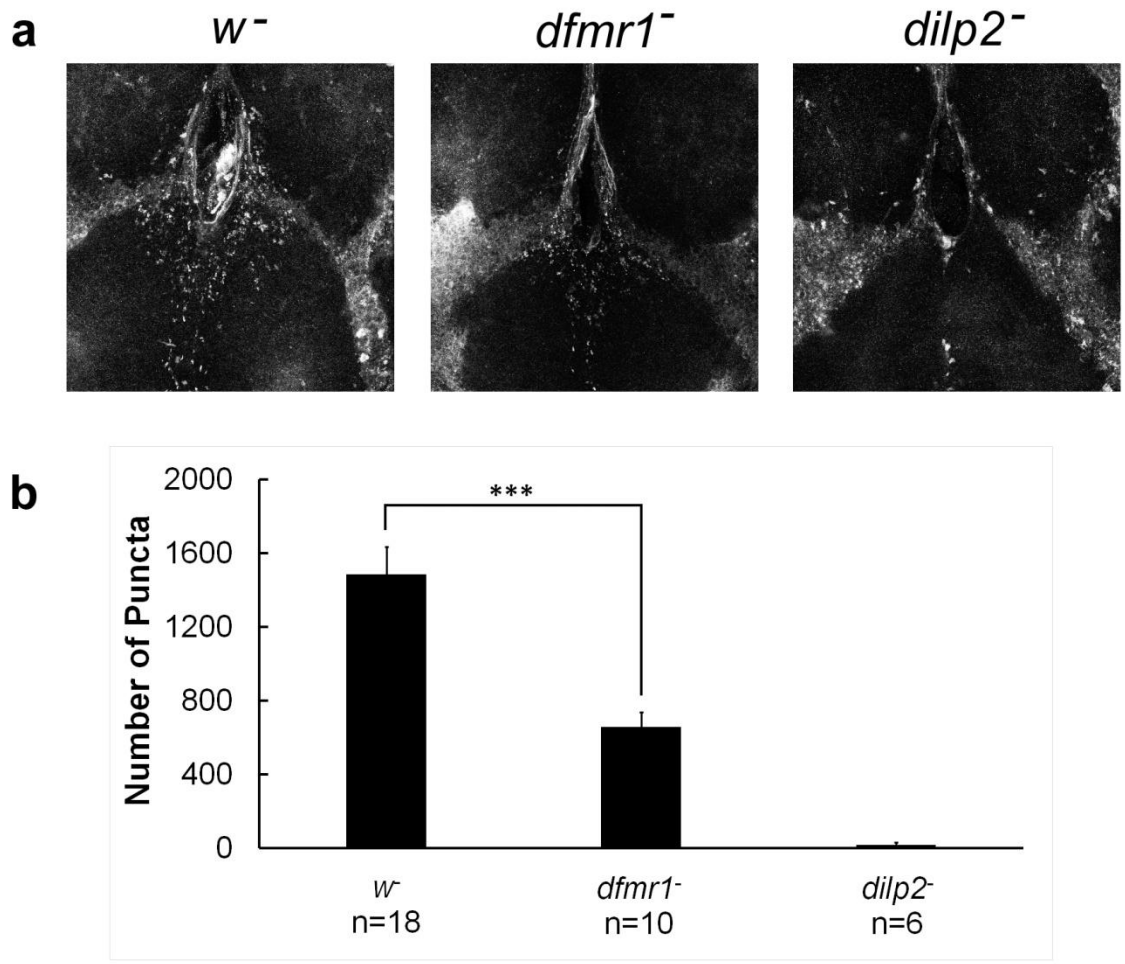

Figure A-5. Quantity of Dilp2 puncta is reduced in the subesophogeal region of *dfmr1* mutant brains. Brains were dissected from flies, and stained for α-Dilp2. Brains were imaged on the confocal then number of puncta was counted with genotypes blinded. **(a)** Dilp2 staining and **(b)** quantification reveals *dfmr1* mutants have a decreased number of Dilp2 puncta in the subesophogeal region of the brain. Statistical significance was determined by a Kruskal-Wallis test with a Dunn's post-test. Statistical significance is denoted with asterisks (*p<0.05, ** p<0.01, ***p<0.001). Graphs display mean ± s.e.m.

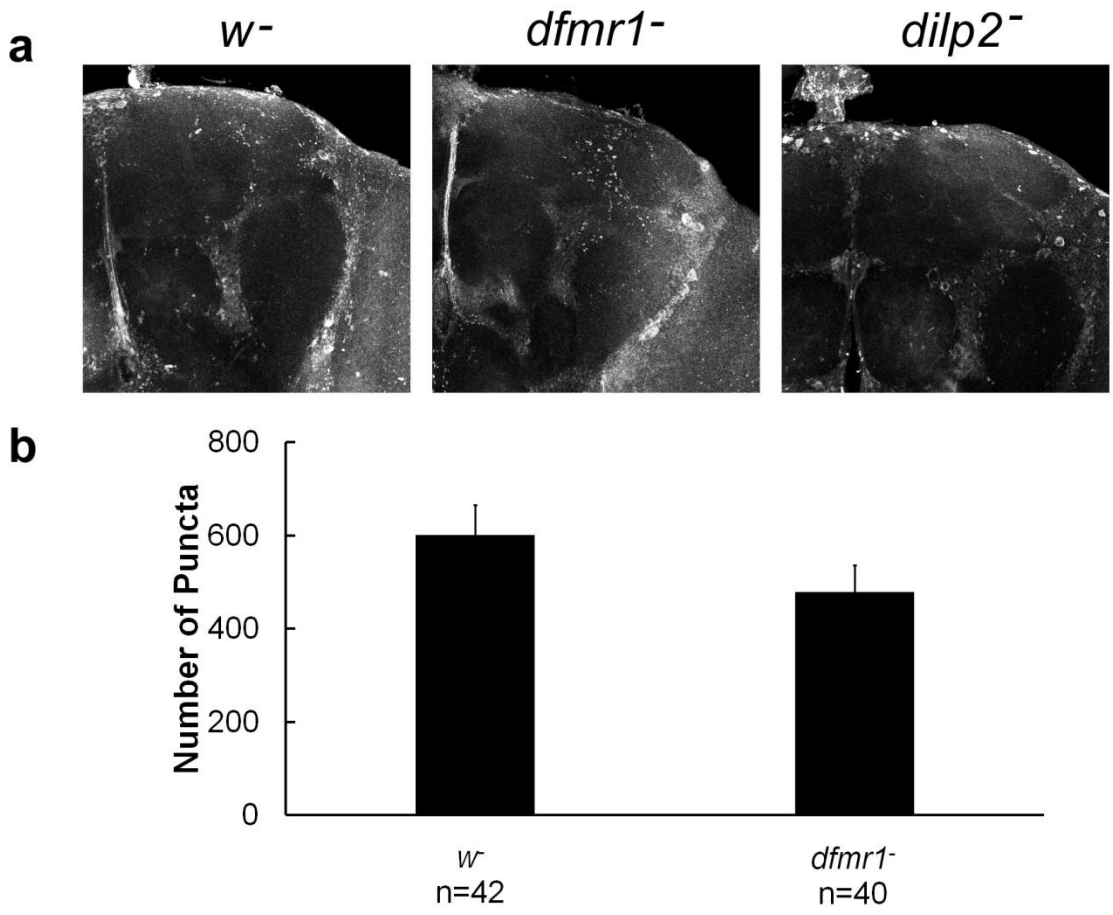

Figure A-6. Loss of *dfmr1* expression does not affect number of Dilp2 puncta dorsal of the subesophogeal region. Brains were dissected from flies, and stained for α-Dilp2. Brains were imaged on the confocal then number of puncta was counted with genotypes blinded. **(a)** Dilp2 staining and **(b)** quantification reveals no change in Dilp2 puncta quantity between *dfmr1* mutant and wild-type control flies in the supraesophageal region of the brain. Statistical significance was determined by a Kruskal-Wallis test with a Dunn's post-test. Statistical significance is denoted with asterisks (*p<0.05, ** p<0.01, ***p<0.001). Graphs display mean ± s.e.m.

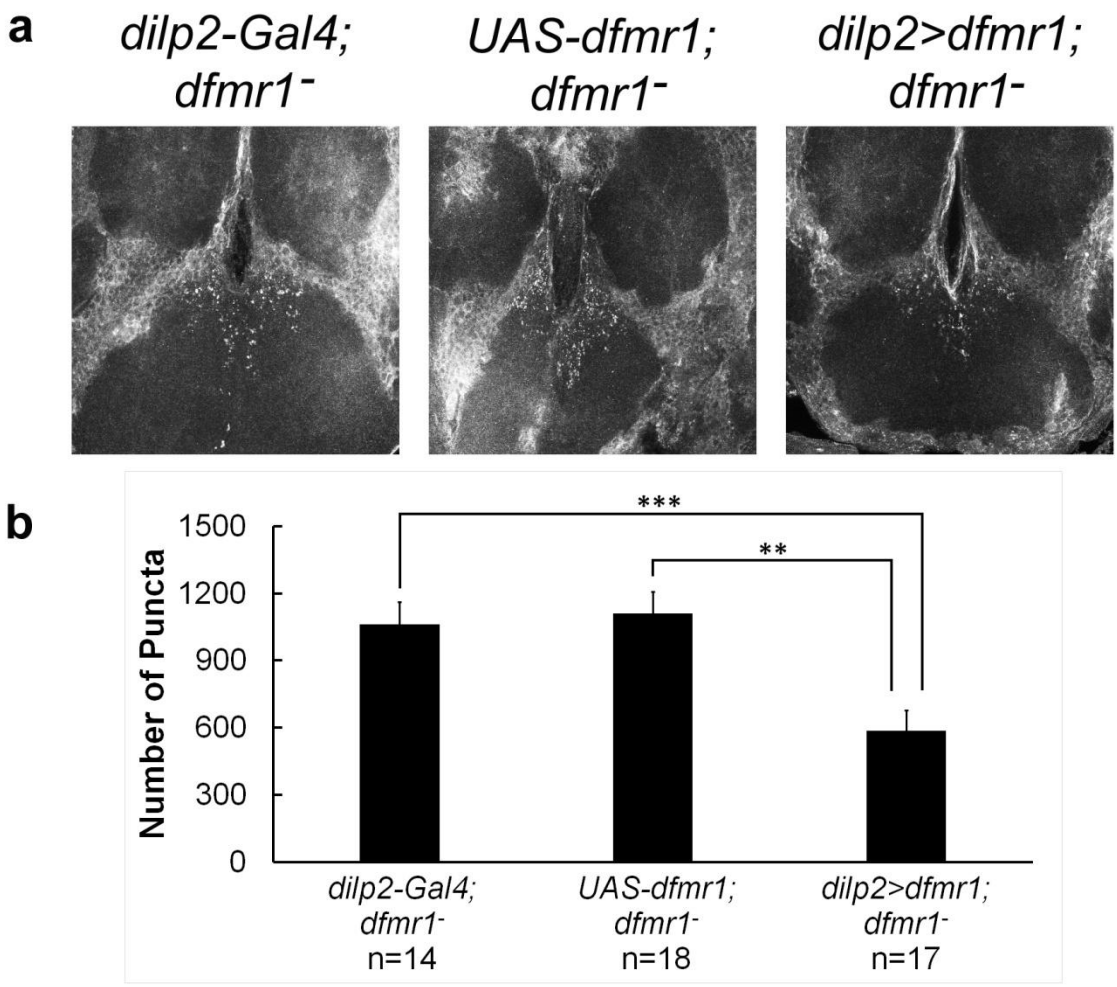

Figure A-7. Expression of *dfmr1* in the IPCs significantly reduces quantity of Dilp2 puncta in the subesophogeal region. Brains were dissected from flies, and stained for α-Dilp2. Brains were imaged on the confocal then number of puncta was counted with genotypes blinded. **(a)** Dilp2 staining and **(b)** quantification reveals that expression of *dfmr1* in the IPCs significantly decreases the number of Dilp2 puncta in the subesophogeal region. Statistical significance was determined by a Kruskal-Wallis test with a Dunn's post- test. Statistical significance is denoted with asterisks (*p<0.05, ** p<0.01, ***p<0.001). Graphs display mean ± s.e.m.

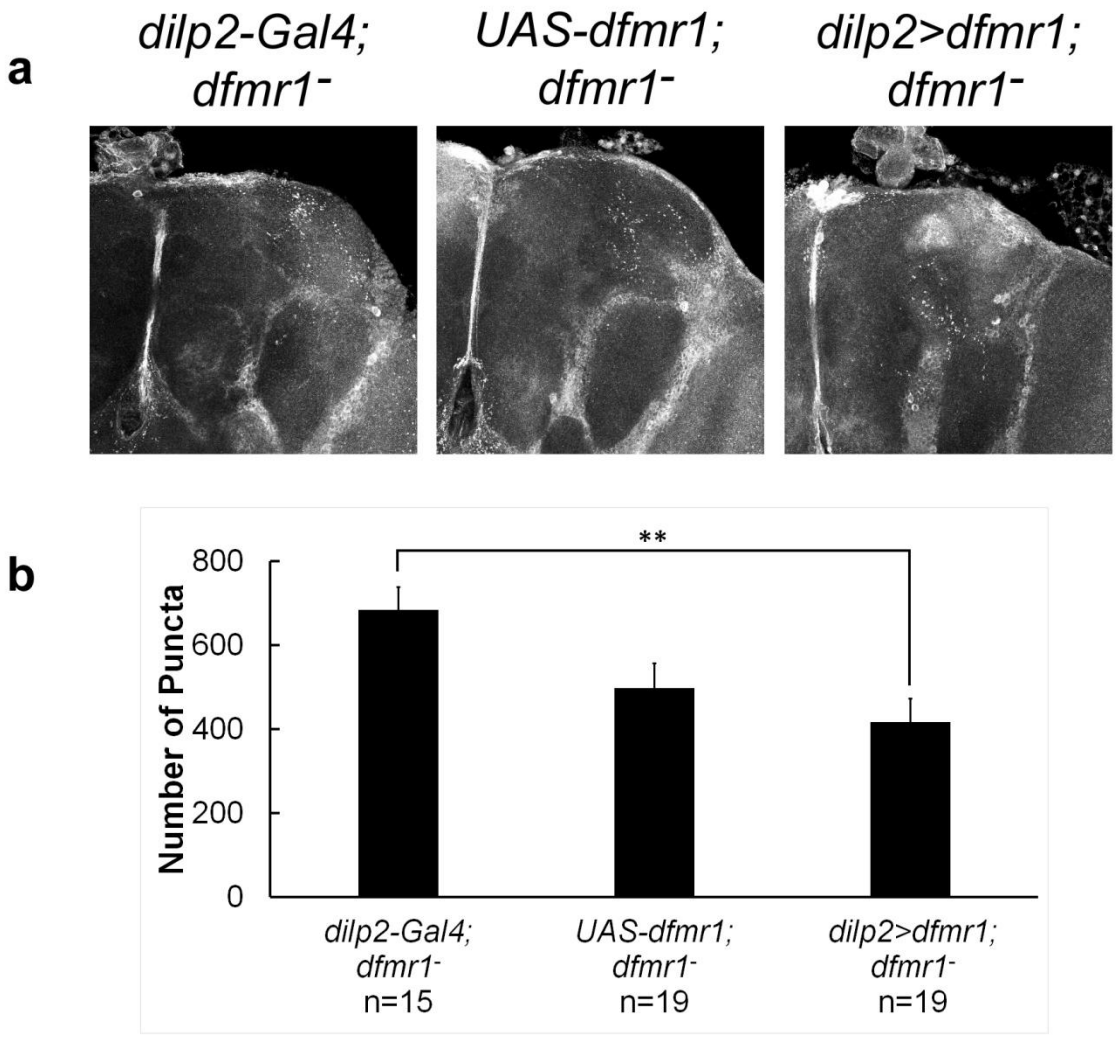

Figure A-8. Expression of *dfmr1* in the IPCs of *dfmr1* mutants reduces Dilp2 puncta in the supraesophageal region. Brains were dissected from flies, and stained for α-Dilp2. Brains were imaged on the confocal then number of puncta was counted with genotypes blinded. **(a)** Dilp2 staining and **(b)** quantification reveals that expression of *dfmr1* in the IPCs reduces Dilp2 staining in the supraesophageal region. Statistical significance was determined by a Kruskal-Wallis test with a Dunn's post-test. Statistical significance is denoted with asterisks (*p<0.05, ** p<0.01, ***p<0.001). Graphs display mean ± s.e.m.

Materials and Methods:

Fly stocks and maintenance

Fly stocks were maintained on standard cornmeal-molasses medium except for those used in the high sucrose diet experiment. *Dilp2^{R}-Gal4* and *dilp2^{W}-Gal4*-containing stocks were obtained from Eric Rulifson and Peng Shen (RULIFSON *et al.* 2002; WU *et al.* 2005). Fly strains containing the *dilp2, dilp2-3, dilp3* and *dilp5* mutations were obtained from Bloomington Stock Center (stock numbers 30881, 30888, 30882, and 30884) (GRONKE *et al.* 2010). The *dfmr1^{3}*allele and *WTrescue* are previously described in (DOCKENDORFF *et al.* 2002). Flies used for dietary insulin resistance studies and for Dilp2 staining were outcrossed to *w1118(iso31B)* flies.

Flies used in the dietary insulin resistance assay were raised on the fly food recipe used in (PASCO AND LEOPOLD 2012). Specifically, in 1 L of food, the following ingredients were added: 10g agar, 60g (1X) or 300g (5X) sucrose, 34g yeast, 82.5g cornmeal, 280µL phosphoric acid, 2.5mL propionic acid, 9mL 25% tegosept.

Circadian Behavior Assay

Flies intended for circadian rhythmicity analysis were raised at 25°C on a L:D cycle. Male flies were collected at 0-3 days of age and maintained on standard fly food in an L:D incubator for entrainment. After 3-5 days, individual flies were loaded into 2% agar, 5% sucrose tubes, which were subsequently placed in activity monitors (TriKinetics) and maintained in dark:dark conditions for 10 days.

Data were collected in 5 minute bins and analyzed with Clock Lab software (Actimetrics) to obtain period and rhythmicity values. Rhythmicity was determined by fast fourier transform (FFT) analysis (with rhythmicity defined as a FFT value of 0.01 or more) as well as visual inspection of the actogram and periodogram. Significant differences in average FFT values between genotypes were determined using a Kruskal-Wallis test followed by a Dunn's post-test (GraphPad, InStat). Relative FFT was calculating by dividing the average FFT value of the

depicted genotype by the average FFT value of the wild-type control: Relative FFT = $FFT_{depicted}/FFT_{wild\text{-}type}*100$.

Immunofluorescence

Male flies aged 3-7 days were collected for dissection. Flies were anesthetized with CO_2, then killed in 100% ethanol for 1 minute. Adult brains were dissected in 1X PBS, then placed in a siliconized microfuge tube containing 1XPBS on ice. Once dissections were complete, brains were fixed in 4% PFA for 25 minutes, washed four times in 1XPBT (.3% Triton-X) for 10 minutes , blocked in 5% Normal Goat Serum diluted in 1XPBT, then placed in the primary antibody solution diluted in 5% Normal Goat Serum overnight at 4°C. The following day, brains were washed four times in 1X PBT and placed in secondary antibody for 3 hours at room temperature, washed four times, then mounted in a 1:5 mixture of Prolong Gold (Invitrogen, P36930) and glycerol with 2% N-propyl gallate (Sigma, P3130).

Primary antibodies used were: anti-Fasciclin II (Developmental Studies Hybridoma Bank, ID4). Anti-Dilp2 was a kind gift from the Sehgal lab.

Mushroom Body Analysis

Images of the mushroom bodies were taken with a Leica TCS SP microscope at 40X with the settings kept constant for all images. Z-stacks were taken to encompass the entire β-lobe region. Slides were imaged blinded to genotype. Following imaging, crossover of mushroom bodies was scored still blinded to genotype.

Dilp2 Staining Analysis:

Brains stained with Dilp2 were imaged on a Leica SP8 confocal with constant settings used for all brains imaged. Stacks were taken of the entire brain either in the subesophogeal region, or on either side of the IPCs. Following imaging, stacks were exported into ImageJ and assigned a random number so genotype was unknown. After blinding, portions of the stack not

including Dilp2 puncta were removed, and the remaining stack was condensed into a maximum projection. Dilp2 puncta were counted on each maximum projection using the Cell counter tool.

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Appendix 2: Protocols

Fly Food—Full Recipe from Jongens Lab

1) Start heating **2000 mL H_2O** and add **25.94 g agar**

2) Add **510 mL H_2O** to a beaker and mix in **40 g yeast** and **160 g cornmeal**

3) When water begins to boil, turn heat down to 1 and add yeast/cornmeal mixture slowly. Cover pot with tinfoil and let mix for **15 mins**.

4) Mix **157.6 mL molasses** and **250 mL H_2O** in a graduated cylinder and add into pot. Add **70 mL H_2O**. Let mix for **30 mins**.

5) Add **.8 mL phosphoric acid**, **7 mL propionic acid** and **25.2 mL 25% tegosept**. Mix for 3 mins then dispense.

To make Tegosept: Add 50g of tegosept into 200mL EtOH

Modified Fly Food Recipe from Pasco and Leopold Paper

1) Add **750mL ddH_2O** to pot with stir bar. Turn heat to 10 and stir to 5.

2) Add **10g agar** and either **60g (1X) or 300g (5X) sucrose**. Add sucrose slowly so it has time to dissolve. Cover pot with aluminum foil.

3) In a separate container, add **250mL ddH_2O**. Then add **34g yeast** and **82.5g cornmeal** while stirring.

4) Bring liquid to a boil. The food must boil or it will not solidify.

5) Add yeast/cornmeal mixture. Stir before adding so the cornmeal doesn't clump. Turn heat to 1.

6) Cook food for 30 minutes.

7) Add **280µL phosphoric acid**, **2.5mL propionic acid** and **9mL 25% tegosept**. Wear goggles for this step.

8) Allow acids to stir in for 5 minutes, then dispense food into desired containers.

DNA Isolation and PCR for Genotyping Protocol

DNA Isolation:
-Make squishing buffer (SB) solution:
10 mM Tris-HCl pH8
1 mM EDTA
25 mM NaCl
200 ug/mL Proteinase K (add later)

1) Add 10µL of 20mg/mL proteinase K to 1mL of squishing buffer (SB) solution and store on ice

2) Freeze 1-2 flies of desired genotype on dry ice in blue 1.5 mL microcentrifuge tubes

3) Add 50µL of SB (with proteinase k added) to each tube and homogenize with blue Kontes pestle (these can be reused if washed in 50% bleach, then ddH_2O, then autoclaved)

4) Incubate tubes at 37°C for 30 mins

5) Incubate at 95°C for 3 mins to inactivate proteinase K

6) Spin tubes for 2 mins at 8,000 rpm and store at -20°C until PCR

PCR:
-Dilute primers by adding 10µL ddH_2O per 1 nmol primer powder
-Make 10uM working dilution by making 1:10 dilution (10µL primer + 90µL ddH_2O)

1) Make master mix with following reagents:
17.55µL ddH_2O (18.05µL if no DMSO)
2.5 µL 10x PCR Mix from Invitrogen
.75µL 50mM $MgCl_2$
.5µL DMSO (Optional—use for $dfmr1^3$ primers)
.5µL 10mM dNTPs
.5µL 10uM F. Primer
.5µL 10uM R. Primer
.2µL Platinum Taq Polymerase
(2 µL DNA template)
**DO NOT vortex master mix once Taq polymerase added

2) Add 23µL of master mix to PCR tubes, then add 2µL of appropriate DNA sample (mix and re-spin DNA sample before using)

3) Use following PCR program:
95°C for 5 mins
40 cycles of:

95°C for 1 min
55-60°C for 1 min
72°C for 1 min
72°C for 5 mins
Hold at 4°C

Gel Electrophoresis:
1) Make 1% agarose gel (1g agar in 100mL .5X TBE), microwave, and allow to cool for ~5 mins before adding 4µL Ethidium bromide

2) Allow gel to cool for about 1 hour

3) Add 2µL 10x Loading Dye to PCR product

4) Pipet 20µL of PCR product and 5µL of 100 b.p. DNA ladder into wells

5) Run gel in .5X TBE running buffer at 120 Volts

RNA Isolation Protocol from RNeasy Mini Kit Handbook

*Do not use Buffer RLT and Buffer RW1 with bleach
*Can use up to 30mg of tissue

Pre-Preparation:

1) Add 10uL of β-ME to 1 mL of Buffer RLT (this mixture can be stored at room temp. for up to a month) OR add 20uL of 2M DTT to 1 mL Buffer RLT (can also be stored at room temp. for a month)

2) Dilute Buffer RPE with 96-100% ethanol

3) Prepare DNase I solution (DO NOT VORTEX!): Inject 550uL RNase-free water into DNase I vial and mix by inverting vial. Can remove solution from vial and store in single-use aliquots at -20 for up to 9 months. Do not refreeze after thawing.

Procedure:

*This entire procedure should be performed at room temperature—do not centrifuge at temperatures below 20 degrees
1) Add 350uL Buffer RLT to tissue and homogenize with pestle
2) Centrifuge lysate for 3 min at full speed, then transfer supernatant to fresh tube. (Note that pellet may be very small/invisible).
3) Add 1 volume (factor in loss of volume after centrifuge) of 70% ethanol and mix by pipetting (do not centrifuge). Do not worry if you get precipitate.
4) Transfer up to 700uL of sample, including any precipitate, to a RNeasy spin column/collection tube. Close lid and centrifuge for 15 seconds at more than 8,000 g (10,000 rpm). Discard flow-through and reuse collection tube.
5) DNase Digestion (optional): *DO NOT VORTEX DNase I*
*Have to use their special DNase digestion buffer because normal DNase digestion buffers not compatible with on-column digestion

A) Add 350uL Buffer RW1 to spin column. Close and centrifuge for 15 seconds at more than 8,000 g. Discard flow-through and reuse column.
B) Add 10uL DNase I solution to 70uL Buffer RDD. Mix by gently inverting tube and briefly spin down. Add all 80 uL to spin column and incubate on bench for 15 mins
C) Add 350uL Buffer RW1 to spin column and spin for 15 seconds at more than 8,000 g. Discard flowthrough.

6) Add 500uL Buffer RPE to spin column and centrifuge for 15 sec. at more than 8,000 g. Discard flow-through and reuse collection tube
7) Add another 500uL Buffer RPE to spin column and centrifuge for 2 mins at more than 8,000 g.
8) (Optional) Transfer spin column to new collection tube (being careful not to let it contact flow through). Centrifuge at full speed for 1 min.
9) Place spin column in a 1.5mL tube and add 30uL to 50uL RNase free water to spin column membrane. Centrifuge at more than 8,000g for 1 min to elute RNA.
10) Can repeat step 9 if yield should be more than 30ug (will result in high RNA yield, but lower RNA concentration).

Invitrogen Superscript III Reverse Transcriptase Protocol

*Can be used for 10pg to 5ug of total RNA or 10pg-500ng mRNA

1) Combine the following in a PCR tube:
    1 uL 50uM oligoI(dT) or 50-250ng random primers
    10pg to 5ug total RNA or 10pg to 500ug mRNA
    1 uL 10 mM dNTP Mix
    ddH_20 for total volume of 13 uL

2) Heat for 5 min at 65°C, then incubate on ice for at least 1 minute

3) Centrifuge briefly, then add the following and mix by pipetting up and down:
    4uL 5x First Strand Buffer
    1uL .1M DTT
    1uL RNaseOUT
    1uL Superscript III RT (or ddH_20 for no-RT control)

4) If using random primers, incubate at 25°C for 5 min

5) Incubate at 50°C for 30-60 mins, then inactivate reaction by heating at 70°C for 15 mins

qPCR Protocol for MX3000 System in Kaestner Lab

1) Put clear MX3000 plate on ice. You can use a pen to mark the plate to ease loading. Take out Brilliant III Ultra Fast Sybr Master Mix kit and allow to thaw in darkness

2) Make master mix of following reagents. Keep in mind that final volume (with cDNA) will be 20µL

2x Brilliant III Ultra Fast Sybr Master Mix	10µL
1mM Rox Reference Dye (1:500 dilution)	.3µL
10µM Forward Primer	.4µL
10µM Reverse Primer	.4µL
ddH_2O	8.2µL
cDNA	1µL

*Note that adding .4µL of primers gives 200nM final concentration. This should be adjusted as necessary for optimum efficiency

3) Load 19µL of master mix into qPCR plate wells. then load 1µL of cDNA sample. Keep plate covered as much as possible if not loading since master mix is sensitive to light.

4) Go upstairs to Kaestner lab to turn on qPCR machine so it has time to warm up. Click on the MxPro icon → select the sybr option → say "yes" to using sybr3. The PCR program run should be the following:

95°C for 3 min
40 cycles of:
    95°C for 5 seconds
    60°C for 20 seconds
    95°C for 1 min
60°C for 30 seconds
95°C for 30 seconds
A) Highlight wells in plate being loaded and select "unknown" for well-type and "rox" and "fam" for dyes being used
B) Click "increase increment" and highlight each of your replicates (so 2-3 wells) and they'll be labeled "1" "2" "3" etc. so it's easy to see which samples are grouped
C) Save plate into Jongens folder
    *When you start setting up the plate, the lamp will begin to warm up (takes ~20 mins). The lamp will not stay on forever, though, so don't take too long to set up your plate.

5) Load 1µL of the cDNA sample into qPCR plate. Make sure to check tip each time so that exactly 1µL is loaded!

6) Bring plate upstairs and spin down in Kaestner centrifuge. Load your plate into the machine, then click "next" and "run." Make sure to click "turn off laser after run" if no one is using machine after you.

7) After run, remove plate and export data in following way:

A) Check to make sure melting curve looks all right.

B) Make sure “treat wells individually” is checked

C) File → export → Excel, name file and save to your jump drive

Western Blot Protocol using Invitrogen System

In Advance:

1) Add 12.5µL 200mM Na_3VO_4 to 25mL of filter sterilized extraction buffer

Protein Extraction:

0) Turn heat block to 100°C and set centrifuge to 4°C.

1) Complete extraction buffer by adding 10µL 100X protease inhibitor cocktail (PIC), 1µL 1M DTT and 10µL .5M EGTA to 1mL of premade extraction buffer with Na_3VO_4.

2) Freeze flies on dry ice, and remove heads by shaking tube. Transfer fly bodies/heads into petri dish (on dry ice) and transfer fly heads into frozen blue 1.5mL tube (suitable for homogenizer).

3) Add 2µL of extraction buffer for each fly head (20µL for 10 heads, etc.) and homogenize. Keep tubes on ice once extraction buffer is added. Spin tubes for 30 seconds at 13,000 rpm in 4°C centrifuge.

4) Remove supernatant and save on ice. Add an additional 2µL/fly head of extraction buffer to tube with pellet and rehomogenize. Spin these tubes and saved supernatant tubes for 5 minutes at 13,000 rpm in 4°C centrifuge. Combine supernatant in fresh tube.

5) Make a master mix of 4X NuPage LDS sample buffer (blue) and 10X NuPage reducing agent (in fridge).

-Add 7.5µL 4X LDS Buffer and 3µL 10X reducing agent per sample (for final volume of 30µL)

-Add 10.5µL master mix to individual tubes

6) Add 19.5µL supernatant to master mix, mix and spin down quickly, then boil at 100°C for 5 minutes. Allow samples to cool then store on ice while setting up gel.

Make sure truly boiled or you could get wiggly bands

Running Gel:

1) Make 1X MOPS Buffer

40mL 20X MOPS

Fill to 800mL with ddH_2O

2) Unwrap precast gel, remove white tape at bottom and gently remove comb. Rinse with ddH_2O, then put in gel box with writing on gel facing forward (large plate should be facing outside of gel box and small plate should be facing inside).

3) Fill gel box with 1X MOPS buffer, then add 500µL NuPage antioxidant to inside chamber (space between two gels or gel and mock gel).

4) Quick spin samples down, then load 25µL of supernatant (don't disturb pellet). Load samples from right to left since transfer will put them in reverse order. Load 5µL ladder as well.

5) Run gel at 200V for 1 hour (you can increase time by decreasing volts).

Transfer:

1) Make 1X Transfer Buffer
   - 30mL 20X Transfer Buffer
   - 60mL methanol
   - 600µL NuPage Antioxidant
   - Fill to 600mL

2) Fill 3 containers with methanol, ddH_2O and 1X Transfer Buffer and soak P-immobilon membrane:
   - 15 seconds in methanol
   - 2 mins in ddH_2O
   - 5+ mins in 1X Transfer Buffer

3) Soak 6 blotting pads and 2 pieces of filter paper (cut to size of gel) in 1X Transfer Buffer. Flip and squeeze to remove air bubbles.

4) Once gel has finished running, remove it from gel box and place upside down on piece of saran wrap (so smaller piece of plastic is facing upwards).

5) Remove plastic covering gel and cut off wells, taking care to push them away from gel so they don't interfere with transfer. Then put soaked piece of filter paper over gel and smooth down to remove air bubbles.

6) Flip gel over and remove top piece of plastic by placing knife through the slot and gently pushing gel off. Once plate is removed, cut off foot of gel, and gently place membrane on gel (don't touch with hands) and smooth down). Place soaked filter paper on top of membrane and smooth down.

7) Put three soaked blotting pads in transfer box. Move gel/membrane/filter paper stack on top **without flipping it over.** Put two soaked blotting pads on top and put transfer box into new clean gel box while squeezing together. Do not stop squeezing until transfer box is firmly in place.

8) Add 1X Transfer Buffer to inside of transfer box until everything is covered. Fill the outside of gel box with ddH_2O.

9) Add gel box lid and transfer at 45V for 1 hour (this time/voltage cannot be changed).

Staining Day 1:

1) Remove membrane from box and block in 5% milk (in TBS-T--.1%Tween) for 1 hour at room temperature.

2) Wash in 1° antibody overnight at 4°C on rocker.

Staining Day 2:

1) Wash membrane in 1X TBS-T (.1% Tween) for 10 mins x 4

2) Incubate at room temperature in 2° antibody for 2-3 hours

3) Wash membrane in 1X TBS-T for 10 mins x 4.

4) Mix equal amounts of the ECL substrate chemicals together and incubate on blot for 5 mins. (Use West Pico kit for easy to detect proteins and Lumi Light kit for more sensitive detection).

Brain Dissection and Immunohistochemistry Protocol

Preparation of 4% PFA:
1) Dilute 16% PFA with 1xPBS to make enough 4%PFA for each of your samples (450µL/sample)

Dissection:
1) Anesthetize flies with CO_2, double-check genotype, then brush flies into petri dish filled with 100% EtOH and swirl for approximately 30 seconds.

2) Carefully dump off EtOH (without losing flies and using Kim-wipe to remove additional EtOH), then add 1xPBS to petri dish and begin brain dissection

3) After dissecting each brain, move it to a .5mL microfuge tube containing 400µL 1xPBS on ice. Do not dissect for more than 1.5 hours before fixing. Ideally fix brains within 30 minutes.

Immunohistochemistry:
1) Spin tubes down quickly in centrifuge to pellet brains at bottom, then remove 1xPBS with pipette and add 450µL 4%PFA. If staining for GFP, cover brains with foil from now until the end of the procedure.

2) Rock brains with fix for 25 minutes at room temperature on nutator.

3) Quick spin brains and remove PFA (do this in hood), then wash with 400µL 1xPBT (.3% Triton-X) for 10 mins x 4. Rock on nutator during each wash.

4) Block brains in 400µL of 5% Normal Goat Serum (NGS) diluted in 1xPBT for 1 hour at 4°C. Rock in nutator in cold room.

5) Wash brains overnight at 4°C in 1° antibody solution diluted in 5% NGS (make new solution—don't just add antibodies to blocking solution).

6) Wash brains in 1xPBT (.3% Triton-X) for 5 mins x 1, then for 15 mins x 4 at room temperature. (washing amount may vary).

7) OPTIONAL: Block brains for 30 mins in 5% NGS at room temperature. Do this if staining for mushroom bodies.

8) Wash brains in 2° antibody solution diluted in 5% NGS for 2-3 hours at room temperature. If not using foil already, cover brains with foil from this step onward. Secondary antibodies are light sensitive!

9) Wash brains in 1xPBT for 5 mins x 1, then for 15mins x 4 at room temperature.

Mounting Brains:
1) Prepare mounting media by making 1:5 solution of Prolong Gold (from Invitrogen) in "special" glycerol solution with 2% n-propyl gallate. (10µL Prolong Gold + 40µL glycerol mixture for total volume of 50µL). ***Before diluting allow Prolong Gold to warm up for at least 20 minutes.

2) Remove as much PBT as possible from brains using P-10 pipette if necessary. Add 20µL mounting media, watch brains float, and VERY SLOWLY suck up mounting media and brains, then pipette VERY SLOWLY onto slide

3) Arrange brains into lines with forceps or pipette tip. Add 5-10µL additional mounting media, then put on coverslip.

4) Keep slides at 4°C overnight , then seal with nailpolish. Let nailpolish dry and move slides to -20°C.